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STUDIES ON CANINE PARVOVIRUS INFECTION

by

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Thesis submitted for the degree of Doctor of
Philosophy in the Faculty of Veterinary Medicine,
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**TEXT
BOUND INTO THE
SPINE**

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SUMMARY

=====

In 1978, two new disease syndromes in dogs were recognised almost simultaneously in America, Australia and Europe. The first was one of sudden death due to heart failure in young pups as a result of a non-suppurative myocarditis. The second was vomiting, diarrhoea or dysentery and death in weaned pups and adult dogs as a result of a severe enteritis, with lesions similar to those seen in feline parvovirus infection (feline panleukopaenia/feline infectious enteritis) in cats. Both syndromes were rapidly associated with a distinct canine parvovirus closely related to feline parvovirus. Retrospective serological surveys have shown that the new dog virus (canine parvovirus - CPV) was not present in the canine population before 1978.

The studies described in this thesis record a series of investigations into the pathogenesis of the enteric disease caused by infection with CPV in susceptible dogs. Chapter 1 reviews the features of naturally occurring CPV infection in dogs, discusses the relationship between CPV and other parvoviruses, particularly feline parvovirus, and surveys the experimental studies of CPV infection already carried out. Chapter 2 describes the techniques used in subsequent investigations.

A preliminary experiment, described in Chapter 3, showed that oral infection with CPV grown in tissue culture could cause enteric disease and was transmitted to in-contact dogs. However, not all infected dogs developed clinical disease. Histological examination revealed changes in lymphoid tissues of all infected dogs but intestinal lesions only in those with clinical enteric disease.

Following this preliminary study, a novel approach to the production of high quality challenge material was undertaken (Chapter 4) and the innate physical resistance of CPV used to allow extraction of large amounts of infectious, purified virus from faecal material. In addition, immunocytochemical techniques were developed (Chapter 5) to allow the localisation of viral antigens in tissues following infection with CPV. Immunofluorescence techniques proved a rapid and simple method of screening tissues for antigen while immunoperoxidase techniques allowed accurate localisation of viral antigens in tissue sections.

In Chapter 6, oral infection of dogs with CPV of faecal origin resulted in severe clinical enteric disease with, in some dogs, dehydration, dysentery and death 5 to 7 days after infection. Infection again resulted in widespread destructive lymphoid lesions but there were also severe small intestinal lesions. In the intestines the initial change was destruction of epithelial cells in the proliferative zone of the intestinal crypts with shortening of villi. This progressed to complete loss of villi with dilation of remaining crypts and attenuation of crypt epithelium. In animals which died there was complete collapse of intestinal architecture. Regeneration of crypt and villus structure in surviving dogs was well advanced by 12 and 13 days after infection. The haematological, serological and virological findings were also described.

In Chapter 7, the detailed pathogenesis of enteric disease caused by CPV infection was elucidated by serial killing of infected dogs and application of histopathological, immunocytochemical, virological and serological techniques. Following oral infection, virus was shown to localise initially (from 1 day after infection) in lymphoid tissues with widespread lymphocytolysis. Viral replication in the thymus resulted in thymic atrophy. A non-cell associated viraemia occurred 3 to 4 days after infection and was followed by localisation of virus in the proliferating epithelial cells of the intestinal crypts.

Destruction of these cells then resulted in characteristic alterations in intestinal architecture. The sequential haematological, virological, serological and histological features of the infection were described. In addition, scanning electron microscopy was used to visualise the changes in surface morphology of the intestinal mucosa following infection with CPV.

Finally, in Chapter 8, the clinical, haematological, virological, serological and pathological features of CPV infection are discussed in detail. In particular, the variable clinical disease, reflecting the varying severity of intestinal lesion, which may result from CPV infection is considered. The factors which may be responsible for promoting the development of severe intestinal pathology and severe clinical disease following infection with CPV are discussed.

CHAPTER 1 : INTRODUCTION AND REVIEW OF LITERATURE

=====

Problems associated with infectious diseases in dogs have been known since ancient times. Rabies, the oldest known canine infectious disease, was described by Democritus in the 5th Century BC and Aristotle in 400 BC knew that rabies was transmitted by the bite of a rabid dog. Distemper was well recognised in mediaeval Europe and a lucid clinical description was given by the Spanish writer Gonzalo Argote de Molina in 1580 (Whitney and Whitney, 1953). Jenner (1809) recognised the highly contagious nature of distemper and the difficulty of eradicating the disease from kennels.

The problems which distemper caused in sporting kennels, and the subsequent disruption to the hunting activities of the landed gentry, led to research into this condition in Britain in the first half of this century. The sum of £30,000, raised by an appeal in "The Field" magazine, funded the classic studies of Laidlaw and Dunkin (1926) which conclusively demonstrated the viral aetiology of canine distemper and defined the clinical disease syndrome which it produced.

With the definition of distemper and the introduction of vaccines against it, other important infections resulting in serious disease and death in dogs were recognised and defined: infectious canine hepatitis (Rubarth, 1947); leptospirosis (McIntyre and Montgomery, 1952). In the 1950s, the development of tissue culture techniques also led to the production of safe, effective and reliable vaccines against the important "killer" canine diseases. By the early 1970s, the widespread use of vaccines against distemper, hepatitis and leptospirosis had led to the virtual elimination of these diseases in well managed kennels and well cared for household pets.

In the field of research into canine infections, attention turned to the less serious, sublethal infections. During the 1960s and early 1970s, for example, numerous investigations of acute respiratory tract infections in dogs, reviewed by Appel et al. (1970) and Thompson et al. (1975), were

reported. By the late 1970s, interest in infectious enteric disease in the dog was growing, especially as improved techniques of electron microscopy and virus isolation led to the visualisation and isolation of previously unrecognised agents; a coronavirus, for example, has been associated with non-fatal diarrhoea in dogs (Takeuchi et al., 1976). The role of bacteria in canine enteric disease and its potential public health significance also provoked interest (Blaser et al., 1978; Prescott and Karmali, 1978).

Suddenly, in mid 1978, the steady progress of investigation into the non-fatal infectious canine diseases was interrupted by the startling eruption of two hitherto unrecognised disease syndromes in dogs, both associated with high morbidity and mortality and both recognised virtually simultaneously by independent workers in America (Eugster et al., 1978; Appel et al., 1978; Jezyk et al., 1979), Australia (Kelly, 1978; Huxtable et al., 1979; Kelly and Atwell, 1979) and Europe (Jeffries and Blakemore, 1979; Thompson et al., 1979; Osterhaus, et al., 1980a).

The most dramatic syndrome was one of sudden death due to heart failure in pups four to eight weeks of age. In most instances, entire litters of pups died, often almost literally dropping dead over a period of a few days (Kelly and Atwell, 1979; Thompson et al., 1979; Jezyk et al., 1979) - a clinical presentation unprecedented in the experience of both veterinary surgeons and dog breeders. The other syndrome was a severe enteric disease with vomiting, diarrhoea, dysentery and death in both pups and adult dogs. Haemorrhagic enteritis as a sporadic disease entity affecting individual, usually adult animals had been well recognised in the dog (Burrows, 1977) but the new syndrome was distinguished by its high morbidity and mortality, particularly in young animals, by its obvious highly contagious nature and by its appearance in dogs of all ages (Eugster et al., 1978; Appel et al., 1978; Kelly, 1978).

In animals with the enteric syndrome, the histopathological changes in the intestine showed a strong resemblance to those of feline panleukopaenia (feline infectious enteritis) caused by feline parvovirus (FPV) infection (Appel et al., 1978) and parvovirus particles were visualised in the faeces and intestinal contents from affected dogs (Eugster et al., 1978; Appel et al., 1979b). A parvovirus, designated Minute Virus of Canines (MVC) had previously been isolated from dogs (Binn et al., 1967) but no clinical disease had been associated with this agent and the virus detected in 1978-9 was rapidly shown to be antigenically distinct and unrelated to MVC. (Carmichael et al., 1980).

In pups dying from heart failure, the underlying lesion was a non-suppurative myocarditis with intranuclear inclusion bodies in cardiac myocytes which, on electron microscopy, were found to be composed of aggregates of parvovirus virions. It therefore seemed probable that these new syndromes were in some way related and Hayes and his co-workers (1979a) were able to reproduce the enteric disease by oral inoculation of pups, 4 weeks of age, with homogenates of cardiac tissue from pups with myocarditis. It was therefore apparent that these two disease syndromes, enteritis and myocarditis, were simply different manifestations of infection with the same parvovirus which was rapidly shown to be a hitherto undescribed member of the group of parvoviruses and closely related to FPV, the cause of feline panleukopaenia (Johnson and Spradbrow, 1979).

Parvoviruses are the smallest known DNA viruses, 20-30 nanometers in diameter, with single stranded DNA; they are members of the family Parvoviridae which includes three distinct genera: the Parvoviruses, the Adeno-Associated viruses (AAV) and the Densoviruses. Only one virus is yet recognised in the genus Densovirus, the insect virus of Densonucleosis (Kurstak, 1972). The AAVs require coinfection of host cells by a helper adenovirus in order to replicate; these viruses have been

isolated in tissue culture systems, primarily involving human tissues but also bovine, avian and canine systems (Hoggan, 1970; Hoggan, 1971).

The genus Parvovirus proper encompasses those members of the Parvoviridae which can replicate in mammalian cells without the necessity for coinfection with a helper virus. The genus has been reviewed in detail by Siegl (1976). Briefly, its members share common physiochemical characteristics: the virions have icosahedral symmetry, are unenveloped, ether and heat resistant and band at densities between 1.38 and 1.47 g/ml in Caesium Chloride. Their linear molecule of single stranded DNA is extremely small (only 1.6×10^6 Daltons in the type virus of the genus). This limits the amount of genetic information carried by these viruses and has important effects on viral replication and pathogenicity. Viral replication depends on the use of host cell functions which are expressed only transiently during the synthesis of cellular DNA which occurs primarily in the process of cell mitosis. Consequently, parvoviruses can replicate virtually only in cells which are themselves actively dividing (Siegl, 1976).

There are several well recognised members of the genus Parvovirus. The type virus, Kilham Rat Virus (RV), was the first isolated. This virus is one of a group collectively known as hamster osteolytic viruses since they all produce bone damage when inoculated into neonatal hamsters. The members of this group were originally detected in studies of transplantable tumours of rat (Kilham and Oliver, 1959) and human origin (Dalkorf, 1960; Toolan, 1960a).

The detection of these viruses in association with human tumours resulted in intense interest in and research on their possible role in carcinogenesis. Toolan's group claimed to have found a number of viruses of the hamster osteolytic group in a number of human tumours and tissues from aborted human fetuses (Toolan, 1961a and b; Toolan, 1964). However,

other workers, looking at similar material (Newman et al., 1970), have failed to find such agents and "very few" human sera contain antibody to this group of viruses (Toolan, 1968). In contrast, antibody is widespread in wild and laboratory rat populations (Kilham and Margolis, 1969) and several isolates have been obtained from rats, especially following immunosuppression which appears to convert latent infections into productive infections (Payne et al., 1963; El Dadah et al., 1967).

Since the transplantable human tumours which formed the basis for the studies of Toolan and Daldorf had been produced by serial passage in immunosuppressed rats, it is possible, even probable, that the induction and passage of these tumours had been accompanied by induction of excretion of latent virus with contamination of the tumour tissue. It has therefore been concluded that the hamster osteolytic group of parvoviruses are indeed of rat origin (Ferm and Kilham 1964; Kilham and Margolis, 1969) and this view is now generally accepted (Siegl, 1976).

Although the hamster osteolytic group proved to be disappointing with respect to their original putative role in carcinogenesis, they were extremely important in that the intensive investigations of the group provided the key to the growth of parvoviruses in cell culture. This subsequently led to the isolation of parvoviruses from pigs (Mayr and Mahnel, 1964 and 1966; Cartwright and Huck, 1967) and cattle (Abinanti and Warfield, 1961; Bachmann, 1971) and also to the isolation and definition of the best known of the parvoviruses, the feline parvovirus (FPV) of feline panleukopaenia or feline infectious enteritis.

The clinical disease syndrome of cats now associated with FPV was first described in 1900 by Zsochokke who termed it feline distemper. Zsochokke's description was of a vague illness with anorexia, dullness and occasional vomiting and

diarrhoea; E.coli was isolated from the small intestine. In 1928, Verge and Cristoforini suggested a viral aetiology for the condition since they could reproduce disease following inoculation of animals with bacteria-free filtrates of tissues from affected cats. This was supported by Hindle and Findlay (1932) and Leasure et al. (1934).

The first detailed description of the clinical and pathological features of the disease was by Hammon and Enders (1939a and b) who reported an outbreak in laboratory cats. Typically, animals became anorexic and dull and death occurred rapidly, usually within two days of initial signs. Pyrexia was mild and vomiting and diarrhoea were observed only occasionally. The most striking finding was leukopaenia, observed 3-4 days prior to death and often before the onset of clinical signs. Leukopaenia was most pronounced on the day of death with disappearance of both myeloid and lymphoid cells from the peripheral circulation. There was a wide variation in the severity of clinical signs, some animals being apparently unaffected, but even the "unaffected" animals showed a leukopaenia, albeit less dramatic than in fatal cases. In recognition of this, Hammon and Enders rechristened the disease panleukopaenia which has since been perhaps the most popular name for the condition.

Histologically, there was depletion of the bone marrow and lymph nodes and an enteritis, most severe in the jejunum, with loss of villi; the intestinal crypts were dilated, contained necrotic debris and were lined by flattened epithelial cells. Eosinophilic intranuclear inclusion bodies, providing further evidence of a viral aetiology, were found in lymphocytes in the germinal centres of lymph nodes.

Lawrence and his co-workers (1940) described the haematological changes in more detail showing both a lymphopaenia and a neutropaenia. They also described, to a limited extent, sequential changes in the marrow with a rapid disappearance of mature myeloid cells at the height of the

disease. They suggested the haematological changes could have been due to a secondary factor, "a hypothetical toxic agent", rather than entirely the result of viral replication.

Further accounts of the natural disease appeared during the 1940s. The descriptions by Riser (1943, 1946) of the clinical and pathological features of the infection agreed substantially with the original account of Hammon and Enders (1939a and b). However, Riser observed diarrhoea more frequently and found intranuclear inclusions in intestinal crypt epithelial cells as well as in lymphoid tissues. He also noted a seasonal pattern in fatalities with deaths commonest in autumn in the kittens born earlier in the spring. Torres (1941) implicated insects as potential vectors of the infection, claiming to reproduce disease by permitting fleas from dying animals to infest susceptible ones.

Also during the 1940s, there were several reports of experimental infections of susceptible kittens using filtered tissue homogenates from affected animals (Syverton et al., 1943; Lucas and Riser, 1945; Riser, 1946). Syverton's group infected cats orally, intreperitoneally, intranasally and intragastrically and demonstrated the presence of a transmissible agent in the peripheral circulation two to five days after infection. The appearance and severity of disease produced varied, and this was attributed to the inability to determine the immune status of individuals, so that some cats may have already been immune. More adults than kittens were apparently refractory to infection and this could have been due either to more widespread immunity in adults or to decreasing susceptibility with increasing age.

Lucas and Riser (1945) described in detail the development of intranuclear inclusions in lymph node, spleen and intestinal epithelia following intraperitoneal inoculation of homogenised spleen from sick cats. They observed that inclusions were present in lymphoid tissues before they were

found in the intestines. In a more general description of the same animals, Riser (1946) recorded some sequential histopathological changes and observed that damage to the mucosa of the terminal small intestine preceded damage to the duodenum. An interesting feature of both these publications was the apparent disregard the authors held for the source of experimental animals - "cat 82 was a stray which walked into the laboratory the day the others were received"!

During the 1950s, the pace of research into feline panleukopaenia slowed, with only one report of experimental infection of cats (Newberne et al., 1957) again using tissue homogenates from cats which had died of natural disease: Leukopaenia was the only consistent clinical finding in experimental animals. However, the development of tissue culture techniques in the 1960s saw intense general activity in the isolation of viruses with attempts by workers in Britain and America to isolate the agent of feline panleukopaenia.

In February, 1964, a virus was isolated from the spleen of a leopard which had died of a condition resembling panleukopaenia (Johnson, 1964). Working with this isolate, Johnson (1965a) was able to reproduce the clinical syndrome of feline panleukopaenia by inoculation of domestic cats and concluded that this agent was the cause of feline panleukopaenia (FPL). Johnson (1965b) also observed that the new virus grew better in feline cell culture inoculated before the cultures became confluent and, following extensive tissue culture studies, classified the agent tentatively as a picodna(parvo)virus. (Johnson and Cruickshank, 1966).

Meanwhile, in America, Kilham and Margolis⁽¹⁹⁶⁶⁾ undertook an investigation into cerebellar ataxia in kittens. This common, naturally occurring condition (first described by Herringham and Andrewes (1888)) was very similar to the syndrome of cerebellar hypoplasia which they had induced in neonatal hamsters by inoculation of their RV of the hamster osteolytic

group of parvoviruses (Kilham and Margolis, 1966). They were unable to induce sufficient cerebellar damage to cause clinical effects in kittens by inoculation of RV (Kilham and Margolis, 1965) but were able to demonstrate a parvovirus-like agent, which they termed feline ataxia virus (FAV), in the cerebellum of a naturally occurring case of cerebellar ataxia (Kilham et al., 1971). Inoculation of this FAV isolate into kittens and ferrets resulted in the development of clinically apparent cerebellar hypoplasia (Kilham et al., 1971).

Fortuitously, at this time, Johnson wrote to Kilham, because of the latter's interest in the type parvovirus (RV), concerning his tentatively classified parvovirus (or picodnavirus as they were then termed) of feline panleukopaenia. Similarities between the FAV isolate of Kilham and the FPL isolate of Johnson were immediately apparent and when both groups exchanged isolates and specific sera they were able to demonstrate that the two viruses were, in fact, identical (Johnson, R.H. et al., 1967). When the FPL isolate was inoculated into pregnant cats or directly into foetuses in utero, the resultant full term kittens had cerebellar hypoplasia (Kilham et al., 1967). This finally vindicated the suggestion made by Verlinde (1949) that there was a correlation between an epidemic of panleukopaenia and an upsurge in feline ataxia, a suggestion which had subsequently been ignored.

The virus of feline panleukopaenia/feline infectious enteritis/feline ataxia has since been definitively classified (Johnson et al., 1974) as a parvovirus, feline parvovirus (FPV), and the in vitro studies of Flagstad (1973, 1975) have demonstrated its inherent requirement for actively dividing cells to sustain replication.

Following the isolation of FPV, experimental infections using the purified virus, as opposed to the earlier tissue homogenates, were undertaken. These studies, which defined the pathogenesis of the feline panleukopaenia/enteritis syndrome, were reviewed and interpreted by Kahn (1978). In summary, infection with FPV results in viral replication in the lymphoid system of all susceptible animals. In germ-free animals, only lymphoid lesions are invariably present (Rohovsky and Fowler, 1971) with thymic atrophy the only consistent finding post mortem (Rohovsky and Griesemer, 1967). Localisation of virus in the intestinal epithelium follows lymphoid replication and the severity of intestinal lesions may vary widely (Larsen et al., 1976). The work of Carlson and co-workers (1977a and b) indicated that the severity of intestinal lesions was dependent on the mitotic rate of the cells in the proliferative zone of the intestinal crypt epithelium. They demonstrated that there was more mitotic activity in the crypts of conventional than of germ-free cats, termed this increase in mitotic rate "physiological inflammation" and attributed it to the intestinal flora of the conventional animal. There was, apparently, a direct correlation between the severity of intestinal lesions and the numbers of mitotic cells present in the intestinal epithelium. The intestinal lesions, when produced, were identical to those described by earlier workers, with loss of villi, crypt dilation and intranuclear inclusion bodies in some intestinal crypt cells.

Infection of neonatal kittens with FPV (Csiza et al., 1971a and b) also results in widespread replication in lymphoid tissues and may induce intestinal lesions but infection at this time also results in viral replication and destruction in the granular layer of the cerebellum with resultant cerebellar hypoplasia. The susceptibility of the cerebellum of neonatal kittens to infection is directly correlated with the high mitotic rate found in this tissue at this age (Altman, 1967).

In the 1940s, an epizootic of fatal enteritis occurred in mink ranches in Canada (Schofield, 1949). Affected mink died rapidly after a short period of anorexia and diarrhoea of varying severity. Wills (1952) suggested a similarity between the agent responsible for "mink enteritis" and the agent of feline panleukopenia since serum from recovered cats protected mink against illness following inoculation of mink tissue homogenates which produced typical disease in littermates. Further, Macpherson (1956) demonstrated a leukopaenia in experimental mink enteritis and was able to reproduce the syndrome in mink by inoculation of tissue suspensions from a cat with panleukopaenia. Subsequently, virological comparisons of isolates of mink enteritis virus (MEV) and feline panleukopaenia (FPV) demonstrated that these viruses were serologically indistinguishable (Johnson, 1967). However modern restriction-mapping techniques have demonstrated a slight difference in the DNA genomes of FPV and MEV isolates (Tratschin et al., 1982).

The sudden epizootics of mink enteritis in the 1940s are, in some respects, paralleled by the sudden emergence of disease in dogs associated with a parvovirus in 1978-9. Similarly, the occurrence of two distinct disease syndromes in association with this "new" canine parvovirus is mirrored in the two distinct clinical entities, feline infectious enteritis/panleukopaenia and cerebellar hypoplasia, which may result in cats infected with FPV.

The parvovirus responsible for the canine epizootics of 1978-79 was not the first member of the group to be isolated from dogs. Over 10 years previously, Binn's group (Binn et al., 1967) isolated a small DNA virus from rectal swabs and faeces of dogs. No clinical disease has been associated with this agent, designated Minute Virus of Canines (MVC) although antibodies to the virus were present in over 80% of adult dogs (Binn, et al., 1970). MVC is now accepted as a distinct parvovirus (Siegl,

1976). A canine adeno-associated parvovirus (CAAV) has also been demonstrated in canine hepatitis virus stock (Ward and Tattersall, 1978).

CAAV is not, by definition, a member of the Parvovirus genus but only of the wider, Parvoviridae family while Binn's MVC has been shown to be antigenically distinct and unrelated to the "new" canine parvovirus of 1978-79 (Carmichael et al., 1980). There is now little doubt that the "new" virus of the 1978-79 epizootics is an autonomous canine parvovirus (CPV) and the characteristics of this new virus, as far as they have been established, have been reviewed by Carmichael and Binn (1981).

CPV has been isolated in a variety of cell systems including feline whole embryo cells (McCandlish et al., 1979; Walker et al., 1979), feline kidney cell lines (Black et al., 1979; Horner et al., 1979; Johnson and Spradbrow, 1979), canine kidney cell lines (Gagnon and Povey, 1979; Osterhaus et al., 1980a) and embryonic canine lung (Johnson and Spradbrow, 1979). All successful attempts at isolation have involved the inoculation of cell cultures during a period of rapid proliferation of the cells, in suspension, at seeding, or as a preconfluent monolayer. The growth of CPV in cell culture, especially at low passage, can be unreliable with variable yields of virus being produced under apparently constant conditions (Cornwell, personal communication); but the virus can be adapted to specific cell lines growing more reliably and to high titre (Carmichael et al., 1981). Carmichael and his coworkers were able to demonstrate the formation of small plaques in highly passaged cells of the canine, A-72, cell line. These plaques became larger with high passages of the virus. However, most workers have found little obvious cytopathic effect apart from the formation of intranuclear inclusion bodies (Gagnon and Povey, 1979; Johnson and Spradbrow, 1979; Walker et al., 1979). Viral replication in tissue culture is also demonstrable by immunofluorescence (Black et al., 1979;

McCandlish et al., 1979; Osterhaus et al., 1980) and by the detection of viral specific haemagglutinin in infected cultures (Horner et al., 1979; Johnson and Spradbrow, 1979).

The ability to agglutinate mammalian erythrocytes is a property of all autonomous parvoviruses. CPV has been shown to agglutinate porcine, macaque, equine and feline erythrocytes (Carmichael et al., 1980). This has permitted the rapid development of economical, consistent and simple haemagglutination (HA) tests for the detection of viral antigen and rapid and simple haemagglutination-inhibition (HAI) tests for antibody.

Serological tests have demonstrated a very close relationship between CPV and FPV and this has been reviewed briefly by Carmichael and Binn (1981). However, differences between CPV and FPV do exist and isolates have been differentiated by immunodiffusion (Flower et al., 1980), plaque neutralisation and HAI tests (McMaster et al., 1980). Moreover, CPV and FPV differ in the species of erythrocytes which they will agglutinate and in the conditions of temperature and pH required for agglutination (Appel et al., 1979a; Gagnon and Povey, 1979; Johnson and Spradbrow, 1979; Carmichael et al., 1980; Carmichael and Binn, 1981).

Application of modern techniques of molecular virology have confirmed the close relationship but non-identity of CPV and FPV. McMaster et al. (1980) found a close homology, on restriction enzyme mapping, between CPV and FPV, with 86% of mapped sites identical. Tratschin et al. (1982) also showed a close relationship, with most isolates tested differing at 8/112 mapping sites. In contrast, FPV and MEV differed at only 1/112 mapping sites (Tratschin et al., 1982).

The sudden appearance and apparently simultaneous worldwide recognition of CPV differs strikingly from the usual patterns associated with new infectious diseases. First, retrospective serological surveys have confirmed that CPV is indeed a new virus in the dog population. Specific antibody to the virus has not been demonstrated in canine sera prior to 1978 in America (Carmichael et al., 1980), Australia (Walker et al., 1980) or Britain (Macartney, unpublished work). Most "new" infections are simply newly recognised infections which have been detected for the first time as a result of improved techniques. Second, most infectious diseases, even the most contagious, spread in a recognisable pattern with pauses due to natural and artificial barriers. CPV differed from this in its simultaneous recognition in at least three continents, America, Australia and Britain, with antibody becoming prevalent in all three in midsummer 1978 (Carmichael et al., 1980; Walker et al., 1980; Macartney, unpublished observations).

The close relationship between CPV and FPV/MEV has resulted in the suggestion that CPV arose as a result of mutation of FPV/MEV (Johnson and Spradbrow, 1979). Further, the same workers, in an attempt to explain the sudden worldwide occurrence of CPV, postulated that such a mutation could have occurred during laboratory production of vaccines, possibly following contamination of cell lines used in manufacture of canine virus vaccine by FPV with inadvertent selection of a dog adapted mutant. Since many vaccines are distributed worldwide, the mutant virus could then be widely distributed. No evidence of vaccine contamination has been produced although Tratschin et al. (1982) demonstrated that one vaccine strain of FPV differed from CPV at fewer sites than did field isolates of FPV. A less exciting explanation may lie in a combination of the little-known but worldwide traffic in pedigree dogs, often by air, and the extreme resistance of CPV which makes indirect spread of infection extremely common.

Schwers et al. (1979) and Osterhaus et al. (1980b) claim to have detected antibody to CPV in a few canine sera collected in Belgium and the Netherlands in 1977 while Eugster and Nairn in 1977 reported the visualisation of parvovirus-like particles in the faeces of pups with diarrhoea in Texas. However, this virus was not isolated, and antibody analyses of affected pups have not been reported so that it is impossible to say whether or not this outbreak was associated with CPV. It seems likely that the precise origin of this new CPV virus will remain shrouded.

There have been numerous descriptions of the clinical and pathological findings in naturally occurring outbreaks of enteric and myocardial disease caused by CPV.

The enteric syndrome has been the most widely encountered and extensively recorded. The most detailed clinical description is that of Woods et al. (1980) describing outbreaks of enteritis in two large commercial kennels. Anorexia and depression were the first clinical abnormalities observed, followed by vomiting which varied in frequency. Animals were mildly pyrexemic during this early phase. Diarrhoea became evident 6-24 hours after the onset of vomiting and stools varied from slightly mucoid to dark red fluid material. There was often a distinct odour to the faeces, particularly where blood was present. The severity, course and outcome of the disease varied greatly and appeared related to the age of animal: pups of 8-16 weeks were the most severely affected, with only mild signs, requiring little if any treatment, in adult animals.

A similar clinical picture has been recorded by other investigators (Eugster et al., 1978; Fritz, 1979; Harcourt et al., 1980; Jacobs et al., 1980; Kramer et al., 1980; Merickel et al., 1980) who also noted the severity of disease in pups. Meunier et al. (1981), in a detailed epidemiological study following the introduction of CPV into a large commercial

breeding colony, also recorded a high morbidity (approaching 50%) and mortality in pups less than 12 weeks of age with no diarrhoeal disease in adult dogs. Reproductive performance in this kennel was not apparently affected, there being no difference in the numbers of pups born or weaned per litter before and after the introduction of the infection.

Pyrexia has been a widely varying feature in natural outbreaks. Harcourt et al. (1980) recorded pyrexia only occasionally; Woods et al. (1980) noted it regularly; while Appel et al. (1978) found a great variability in the appearance of pyrexia in individual cases.

Reductions in circulating leukocyte counts in affected animals have been recorded (Appel et al., 1978; Eugster et al., 1978; Kramer et al., 1980). Fritz (1979) associated leukopaenia with both neutropaenia and lymphopaenia, recovering animals developing leukocytosis with neutrophilia and monocytosis. Woods et al. (1980) correlated severe leukopaenia with a poor prognosis although Jacobs et al. (1980) found some degree of leukopaenia in many dogs which recovered with treatment.

Post-mortem examination of fatal field cases of enteric disease have revealed lesions predominantly in the small intestine (Nelson et al., 1979; Kramer et al., 1980; Woods et al., 1980; McCandlish et al., 1981; Meunier et al., 1981). Briefly, these investigators have found thickening and inelasticity of the small intestine with varying degrees of serosal congestion and roughening, mucosal haemorrhage and enlargement of mesenteric lymph nodes. Woods et al. (1980) described intestinal mucosal and serosal haemorrhage in association with those areas containing gut associated lymphoid tissue (GALT). Atrophy of the thymus has also been recorded in many reports (Kramer et al., 1980; McCandlish et al., 1981; Meunier et al., 1981). Else (1979) described erosion and ulceration of the gastric mucosa in one dog with enteritis but Nelson et al. (1979) found the stomach in most cases to be

macroscopically normal.

Histologically, the small intestinal changes in field outbreaks have been consistent (Cooper et al., 1979; Fletcher et al., 1979; Nelson et al., 1979; McCandlish et al., 1981; Meunier et al., 1981) and strikingly similar to those described in cats with FPV infection (Hammon and Enders, 1939a; Carlson et al., 1977a). The basic lesion appears to be destruction of epithelial cells in the small intestinal crypts (Cooper et al., 1979) with the jejunum and ileum most often affected (Nelson et al., 1979) although in many cases the entire small intestine has been involved.

Most typically, there is dilation of intestinal crypts with loss of associated villi (Pletcher et al., 1979; McCandlish et al., 1981). Superficial mucosal necrosis has been attributed to autolytic change (Pletcher et al., 1979) and is often associated with bacterial invasion (McCandlish et al., 1981). Dilated crypts are lined by flattened epithelial cells (Nelson et al., 1979) and often contain necrotic debris and neutrophils (Cooper et al., 1979). Sparse, amphophilic to eosinophilic intranuclear inclusion bodies may be seen in intact crypt enterocytes (Cooper et al., 1979), especially in areas with acute epithelial necrosis (Meunier et al., 1981).

In some cases, there is almost complete loss of intestinal epithelium with collapse of the lamina propria (Meunier et al., 1981), only sparse crypts, lined by large irregular epithelial cells, remaining visible. In other cases (Cooper et al., 1979), some crypts are lined by hyperplastic, apparently regenerative epithelial cells.

Colonic involvement has been recorded only occasionally and has been mild, with slight dilation of colonic crypts by small amounts of mucus and cell debris (Nelson et al., 1979). Renal lesions, with tubular epithelial degeneration and necrosis, have been reported by one group (Harcourt et al.,

1980).

Degeneration and necrosis of lymphoid tissues have been found by most investigators, with depletion and necrosis of cortical cells in the thymus and depletion of lymph nodes, GALT and splenic white pulp (Cooper et al., 1979; McCandlish et al., 1981; Meunier et al., 1981). Large reticular macrophage-like or histiocytic cells have been prominent in the germinal centres of lymphoid tissues (Pletcher, et al., 1979). There are no detailed descriptions of bone marrow changes.

A similar clinicopathological syndrome, apparently associated with CPV, has been described in non-domesticated canids: maned wolves (Fletcher et al., 1979), bush dogs and crab-eating foxes (Mann et al., 1980) from South America; coyotes (Evermann et al., 1980) in North America; and raccoon dogs and foxes (Neuvonen et al., 1982) in Norway.

Accounts of the myocardial syndrome are fewer than those recording enteric disease. The emergence of the myocardial syndrome was heralded by the sudden death of pups between 4 and 8 weeks of age (Hayes et al., 1979a; Huxtable et al., 1979; Kelly and Atwell, 1979; Thompson et al., 1979). Deaths in pups of this age were uncommon prior to 1978, and the dramatic syndrome described had not previously been recognised by dog breeders or veterinary surgeons. Pups were found dead, without premonitory signs, or were seen to die rapidly within a few minutes (McCandlish et al., 1981). Post-mortem findings were those of heart failure with pulmonary oedema and hepatic congestion. Histopathological examination revealed a non-suppurative myocarditis with separation of myocytes by oedema and infiltration of mononuclear cells. There was focal necrosis of cardiac myocytes and the presence of large basophilic intranuclear inclusion bodies in some myocytes indicated a viral aetiology (Hayes et al., 1979b; Thompson et al., 1979).

This was confirmed by electron microscopical visualisation of parvovirus-like particles in inclusions seen in ultrathin sections (McCandlish et al., 1979; Hayes et al., 1979a) and in negatively-stained myocardial homogenates (Lenihan et al., 1980). Hayes et al. (1979a) showed that the inclusions stained positively by immunofluorescence using antiserum against CPV and subsequently reproduced the enteric syndrome by oral inoculation of 4 week old pups with homogenates of heart from a pup dying of myocarditis.

Subsequent studies of litters affected by myocardial disease (Atwell and Kelly, 1980; McCandlish et al., 1981) have given a clear picture of the field disease and the variations in clinical pattern which occur. Most strikingly, entire litters of pups were affected although not all died in acute heart failure. Some pups survived the acute phase of viral replication with myocyte necrosis and myocarditis but developed varying degrees of myocardial fibrosis which subsequently resulted in subacute or congestive cardiac failure some time after 12 weeks of age. Some "surviving" littermates have succumbed to heart failure as late as 27 months of age (McCandlish et al., 1981).

Despite the fact that both enteric and myocardial syndromes would appear to be caused by the same virus, in general, pups dying with myocarditis have no destructive intestinal or lymphoid lesions at death (Hayes, et al., 1979a) while most dogs with enteritis do not have cardiac involvement (McCandlish, 1981). Nonetheless, both syndromes may be present in different litters in the same kennel, especially when infection first occurs (Harcourt et al., 1980). Sparse intranuclear inclusion bodies may be observed in the heart muscle of pups dying as a result of CPV enteritis (Jeffries and Blakemore, 1979) and are particularly noticeable in pups dying with enteritis at 5-6 weeks of age (McCandlish et al., 1979).

It has been hypothesised that the occurrence in dogs of the two distinct enteric and myocardial syndromes, like the

occurrence of enteritis and cerebellar hypoplasia in cats, is a consequence of the proclivity of parvoviruses for actively dividing cells and reflects infection in different ages of animals which have varying mitotic activity in different tissues (McCandlish et al., 1979). In the neonatal period, there is mitotic activity at a relatively high rate in heart muscle (Bishop, 1972) but at a relatively low rate in intestinal epithelium: infection at this stage is therefore likely to result in myocarditis. By weaning, cardiac mitosis has ceased but intestinal mitosis (Koldovsky et al., 1966) has increased: infection at this stage will be more likely to result in enteritis, the sparse inclusions observed in cardiac myocytes representing infection of the last few dividing myocardial nuclei. Robinson et al. (1980a) have also suggested a specific tropism of the virus for myocardial cells.

Active cell mitosis may be observed in 2-4% of canine cardiac myocytes immediately after birth, the mitotic rate reducing from 15 days after birth (Bishop, 1972). However, cardiac myocytes are polyploid and intracellular DNA synthesis in such cells may occur postnatally without concomitant mitosis (Rumyantsev, 1977). Since parvoviral replication is dependent on functions expressed during DNA synthesis (most usually associated with mitosis) it would seem likely that the most sensitive estimation of the susceptibility of cardiac myocytes to CPV would involve measurement of DNA synthesis as opposed to simple mitotic activity.

Limited measurements of DNA synthesis using tritiated thymidine have been carried out and the synthetic capability of neonatal cardiac tissue demonstrated (Lenghaus et al., 1980) although detailed data were not presented. These workers were also able to reproduce myocardial disease by inoculation of pups in utero with virus originally isolated from a natural case of myocarditis. Inoculated pups died in acute heart failure with myocarditis at 3-4 weeks of age or, when killed at 12 and 17 weeks, showed myocardial scarring. Oral infection of

neonatal, colostrum-deprived pups has also resulted in the development of myocarditis at 3-4 weeks of age (McCandlish et al., 1981).

Despite the fact that infection of dogs with a pathogenic parvovirus was first recognised only in 1978, the clinical and pathological features of the natural field disease syndromes have, as detailed above, been extensively described. Studies on the biology of CPV and its relationships to other parvoviruses (McMaster et al., 1980; Tratschin et al., 1982) and reports on the development of effective vaccines (Smith et al., 1980; Pollock and Carmichael, 1982a and b) have also been published.

However, in October 1979, at the beginning of the work described in the following Chapters (2-7), only one account of experimental infection of susceptible dogs with Canine Parvovirus (CPV) had been published (Appel et al., 1979a). This study was concerned with the isolation of CPV in cell culture and the subsequent development of an inactivated vaccine from the tissue culture derived virus. The experimental disease was only briefly described, and no pathological data were presented. Oral infection with filtered faecal material from a dog with clinical CPV enteritis, and intravenous infection with virus in its fourth passage in cell culture resulted in a mild lymphopenia and a low grade pyrexia at days 3, 4 and 5 post-infection (PI). There were no clinical signs of enteric disease in any of the twelve dogs infected, nor was there any mention of viral excretion or seroconversion.

The series of experiments described in this thesis was therefore undertaken at that time to establish the detailed sequential pathological changes occurring as a result of infection with CPV, and to relate these to the clinical, serological, haematological and virological features of the infection. This work, of necessity, has been carried out at a time when CPV has been the subject of intense research by groups

in various parts of the world and several reports on experimental CPV infection have been published during the course of these studies.

In 1980, Carmichael et al., in a report on the haemagglutinating activity of CPV, mentioned the oral infection of six-week old puppies with CPV in its second passage in tissue culture. Clinical signs were not described. Using an HA test, virus was detected in the faeces between 3 and 9 DPI, with maximum levels of 10240 at 6 DPI. Serum antibody was first detected using an HAI test at 4 DPI, the level rising sharply to reach a plateau level of 5120 by 8 DPI.

Carman and Povey, in a short communication in 1980, and more completely later (1982a and b), reported the successful reproduction of clinical disease in susceptible dogs. Dogs were fasted for 24 hours before oral infection and for 48 hours afterwards. Also in 1980, Robinson et al., (1980b) reported the intravenous infection of two litters of puppies with CPV; general clinical and pathological features were described. In both the above studies, clinical signs of dullness, mild pyrexia, anorexia, vomition and diarrhoea of varying severity were noted. Although not all infected dogs became clinically affected, the majority of animals in both studies did develop clinical enteric disease. The character of the faeces in affected dogs ranged from slightly soft through mucoid to bloody diarrhoea, with dysentery in the most severe cases. Robinson et al., (1980b) reported clinical signs at 4 and 5 DPI while Carman and Povey noted clinical signs ranging from 1 DPI until 14 DPI. Serological and haematological findings were not presented in either publication.

Pathological features described by both groups of workers were essentially similar, with depletion of lymphoid tissues a consistent feature and characteristic intestinal lesions in clinically affected animals similar to those seen in the field disease. The lesions consisted essentially of loss

of villi, dilation of the crypts and attenuation of the lining epithelium. No sequential pathological examinations were carried out by either group of workers.

In 1981, Potgeiter et al. and Azetaka et al. reported the experimental reproduction of enteric disease following oral infection with CPV. In both reports, clinical signs were noted at 4 DPI, with more severe signs in some of the dogs at 5 DPI. Three out of five dogs in Potgeiter's study developed clinical abnormalities, while one of the two susceptible dogs in the experiment reported by Azetaka was affected. When clinical disease did become apparent, the clinical signs were essentially similar to those described by Robinson et al. (1980b), as were the histopathological features. Azetaka noted a leukopenia in the dog which became severely ill, with the total leukocyte count less than 1×10^9 /litre on the fifth day after infection. In neither report was there any discussion of the sequential pathological features.

Pollock (1982), in a detailed but confused paper on CPV infection, described the clinical, serological and haematological features, together with the pattern of viral excretion. The challenge material used was either virus in its 4th passage in tissue culture or a homogenised suspension of various tissues from a dog challenged oronasally with such virus and killed at 4 DPI. Dogs were challenged either oronasally, intravenously or intramuscularly, although subsequently the intravenously and intramuscularly challenged animals were discussed as a single parenterally challenged group. Clinical signs were similar to those noted by Robinson et al. (1980b) and were found to occur 4-5 DPI if the dogs were challenged oronasally or at 2-3 DPI if the virus was administered parenterally. Antibody first appeared at 5 DPI in the oronasally challenged dogs and at 3 DPI in the parenterally infected group. A relative lymphopenia was noted at 4 DPI in both groups of dogs. The pattern of faecal excretion was similar to that noted by Carmichael et al. (1980), with maximum

amounts of virus detected in the faeces at 5 DPI, with no detectable virus in the faeces by 9 DPI. Faecal excretion of virus was noted 24-48 hours earlier in the dogs which had been infected parenterally. Pollock also reported a plasma viraemia at 3 and 4 DPI in the orally infected dogs, only small amounts of virus being associated with the buffy coat fraction. Only one of the dogs challenged parenterally had a viraemia, possibly a reflection of the rapid rise in serum antibody in these dogs. There was no discussion of the pathological features of the infection in this report.

McAdaragh et al. (1982) were the first workers to kill dogs sequentially after infection with virulent CPV, although only four dogs were involved. The histopathological features in the intestine were similar to those noted by Robinson et al. (1980b) i.e. loss of villi, attenuation of the crypt epithelium and dilation of the crypts themselves. The lymphoid system was briefly described, with cortical depletion of the thymus the major finding. The dogs in this study were infected both orally and subcutaneously. Clinical, haematological and serological features, together with viral excretion occurred on the same timescale as in the parenterally infected dogs of Pollock (1982). This may suggest an equivalence between Pollock's intravenous/intramuscular challenge and McAdaragh's oral and subcutaneous challenge, or it could imply, since the experiment was uncontrolled, that these animals had encountered natural infection a short time before the experimental challenge.

These investigations, which have been reported since the beginning of this study in 1979, have therefore extended our understanding of CPV infection. The detailed histopathological features of the infection are nonetheless still relatively poorly described, with particular regard to the sequential changes and to lesions of lymphoid and myeloid tissues. Moreover no detailed immunocytochemical nor electron microscopical studies of the infection have been recorded.

The experiments in this thesis take the form of a progression in depth of study. The preliminary study (Chapter 3) was designed to establish that infection with CPV could be achieved and to determine the most critical time periods for later detailed studies. The type of challenge material was changed in the light of the results of this initial experiment, and the method of preparation of subsequent challenge material is described in Chapter 4. The clinical signs and the detailed pathology of fulminating CPV enteritis are described in detail in Chapter 6. The development of immunocytochemical techniques is described in Chapter 5, while their application to a study of the 'sequential' pathology of the infection, together with scanning electron microscopical studies, are described in Chapter 7.

CHAPTER 2 : MATERIALS AND METHODS

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Experimental Animals

Commercially reared beagles were used for all experiments. The animals were purchased at between eight and twelve weeks of age directly from a closed breeding colony which was known to be free from CPV infection. Sera from adult breeding stock (150 dogs) had been examined by HAI test, and no specific antibody to CPV had been detected. Pups derived from the adult stock were also shown to have no detectable antibody to CPV. The status of the kennel was monitored regularly. Dogs were housed individually or in small groups and fed twice daily on a commercial dog diet (Chappie, Pedigree Petfoods, Melton Mowbray) and milk substitute. Water was freely available at all times. The concrete pens were thoroughly cleaned, fumigated with formalin vapour and rested before and after use by each batch of dogs.

Challenge Virus

Virus of tissue culture origin was used to infect the dogs in Chapter 3, while virus of faecal origin was used in Chapters 5 and 7. The development of a method of purification of virus from faeces, together with the final procedure is described in Chapter 4. All dogs were infected by the oral route.

Tissue culture virus was originally isolated from a dog with clinical CPV enteritis (McCandlish et al., 1979). Faecal material from the dog was diluted with PBS and clarified by centrifugation at 3000 rpm for 15 minutes. The clarified supernatant was then filtered through a Millipore 25 μ filter, and the filtrate maintained under aseptic conditions. Cells were prepared for infection by first inoculating an eight oz. flat-sided bottle with Feline Embryo A (FEA) cells in Eagle's medium supplemented with 10% Foetal Bovine Serum (FBS), each bottle receiving 2×10^6 cells. After two hours' incubation at

37°C, the medium was removed and 1 ml of filtered virus suspension added. This infecting inoculum was incubated with the pre-confluent cell sheet at 37°C for a further two hours, when it was removed and 20 ml of Eagle's medium with 10% FBS added. The culture was incubated for four days at 37°C after which time it was harvested, rapidly frozen and thawed three times and the HA activity assayed. The virus suspension was aliquoted and stored at -70°C until required.

Passage of the original isolate was performed in a similar way, with the exception that 1 ml of the virus suspension from the previous passage was inoculated into the cell cultures in place of the filtered faecal supernatant. Sixth passage virus produced in this way was used to infect dogs in Chapter 3, where each dog received two ml of virus suspension, with an HA titre of 256 per 0.025 ml.

Clinical Examination

In all experiments, each dog was examined at least twice daily. Demeanour and appetite were noted and rectal temperatures were recorded. The nature of faeces produced was observed and faecal samples or rectal swabs collected. Blood samples for haematological examination, virus isolation studies and serological investigations were obtained.

Haematological Procedures

Blood for haematological examination was removed by either ear vein laceration or venipuncture.

Ear vein laceration was used as the method of collection of blood samples for haematological examination in Chapter 3. The ear was clipped, swabbed with 70% ethanol, allowed to dry, and a stab incision made over the anterior auricular vein. 40µl of the resultant drop of blood was removed, placed in 20 ml isoton (Coulter Electronics, Luton)

and subjected to a total white cell count using a Coulter Counter, model ZF, (Coulter Electronics). In addition, a second drop of blood was removed and used to make a smear which was stained using the May-Grunwald-Giemsa method. A differential white blood cell count was performed using this smear, with a standard 200 cell count, from which total absolute neutrophil and lymphocyte values were calculated.

When blood was removed by venipuncture (Chapters 6 and 7) 1 ml samples were placed in a Brunswick tube coated with Potassium EDTA (Sherwood Medical Industries, Crawley, Sussex) and gently rocked for five minutes. A total leukocyte count was then performed on this sample, again using the Coulter Counter and a differential white cell count was performed as above.

Virus Excretion

The presence of virus in faeces, rectal swabs, tissue culture fluids or in fluids from purification procedures was monitored using a haemagglutination (HA) test.

The test was performed using conical bottomed microtitre plates (Flow Labs, Irvine, Scotland). 0.025 ml of phosphate buffered saline (PBS) was added to each of the 96 wells on the microtitre plate, and 0.025 ml of the test suspension added to well 1 and 12 on each row, eight samples being tested on one plate. Serial 2-fold dilutions of this suspension were then made from well 1 to well 11. Prior to the addition of pig erythrocytes, 0.025 ml of serum with a high titre of antibody to CPV was added to the faecal suspension in well 12, to act as a specificity control. 0.05 ml of a 1% suspension of pig erythrocytes was then added to all wells. In addition, for each test, at least three wells were prepared containing only PBS and erythrocytes, acting as a cell control. Following the addition of the erythrocyte suspension, the plates were gently agitated, and then incubated at 4°C for 3 hours or until there was complete buttoning of the erythrocytes in the

control wells.

The results were read using a magnifying mirror, and the titre of the test sample taken as the reciprocal of the highest dilution where complete agglutination was observed.

Preparation of test samples.

Faecal specimens were prepared for the HA test by first suspending a portion of the faeces in PBS at a dilution of 1/10. This suspension was thoroughly agitated for five minutes and then clarified at 3000 rpm using an MSE centrifuge at 4°C for 15 minutes. The resultant supernatant was removed and heat treated at 56°C for 30 minutes to minimise non-specific agglutinins.

Rectal swabs consisting of plain sterile cotton swabs (Exogen, Clydebank) were treated as follows. Each swab was immersed in 2 ml PBS and agitated for five minutes. The swab was left in this PBS for a further 24 hours, before being discarded and the suspension clarified at 3000 rpm for 15 minutes. The clarified supernatants were heat treated at 56°C for 30 minutes.

Test samples of tissue culture fluids were frozen and thawed three times prior to the test. However, neither these fluids nor virus fractions from purification experiments described in Chapter 4 were heat treated.

Preparation of pig erythrocyte suspension. Whole blood was obtained from pigs at slaughter in a local abattoir, and collected in tubes containing potassium EDTA. At the laboratory, the cells were sedimented by centrifugation at 2000 rpm for 10 minutes and washed three times with large volumes of cold PBS. Following final sedimentation 1 ml of the washed cells were suspended in 99 ml of PBS containing 3% Foetal Bovine Serum (FBS), thereby yielding a 1% suspension. This suspension was stored at 4°C until required.

PBS.

This was prepared by dissolving one Dulbecco A PBS tablet (Oxoid, Basingstoke) in 100 ml of water which had been distilled in a glass distillation unit.

Virus Isolation

In Chapter 7, virus isolation in tissue culture was used to demonstrate the presence of virus in serum samples, it being assumed that small amounts of virus would not be detectable by the HA test. FEA cells were seeded into roller tubes in growth medium, each tube being inoculated with 1×10^5 cells. The tubes were stoppered and incubated at 37°C for two hours, when the medium was discarded. 0.1 ml of the test serum was then added to the partially formed monolayer and incubated at 37°C for two hours after which a total volume of 1 ml of Eagle's medium with 10% FBS was added to each tube and the culture incubated at 37°C for four days.

On the fourth day, the tube was frozen and thawed three times and the supernatant checked for haemagglutinating activity using the HA test.

The growth medium consisted of Eagle's Minimal Essential Medium (Gibco Europe Ltd., Uxbridge, Kent) supplemented with Essential Amino Acids (Gibco) and 10% FBS (Gibco). In addition, antibiotics were added at the following concentrations:-

Penicillin	100 mg/litre
Streptomycin	100 mg/litre
Fungizone	30 mg/litre

Serological Procedures

The presence of antibody to CPV in serum samples and in faecal material was monitored by the Haemagglutination-inhibition test (HAI). The test was performed using conical bottomed microtitre plates (Flow Labs, Irvine). 0.025 ml of PBS was placed in each well of the plate and 0.025 ml of test sample added to wells 1 and 12 of the same row, eight samples being assayed per plate. Two-fold dilutions were then made from wells 1 to 11, and 0.025 ml of virus suspension with an HA titre of 8 added, although no virus was added to well 12. Following incubation at room temperature for 60 minutes, 0.05 ml of 1% pig erythrocyte suspension was added to each well, the plate agitated gently and incubated at 4°C for at least 12 hours. To ensure consistency in each test there was a cell control with wells containing only erythrocytes and PBS, a virus control, consisting of an HA test of the antigen suspension, and an HAI test of a serum with a known titre.

The test was read with a magnifying mirror when there was complete buttoning of the cell controls. The titre of the sample was taken as the reciprocal of the highest dilution of the sample which completely inhibited haemagglutination.

Preparation of test samples. Both sera and faecal material were assayed for antibody to CPV using the HAI test. Prior to being subjected to the test, sera were absorbed with a 50% suspension of pig erythrocytes and heated to 56°C for 30 minutes. Faecal material was similarly heat treated, although not absorbed with pig erythrocytes. The absorption with pig cells was performed by first adding 0.4 ml of ice cold PBS to a glass centrifuge tube, followed by the addition of 0.5 ml of test serum and 0.1 ml of a 50% suspension of pig erythrocytes. After gentle agitation to mix the contents, the tube was incubated at 4°C in an ice tray for 20 minutes, the erythrocytes sedimented by centrifugation, and the absorbed serum, now diluted 1:2, decanted.

Faecal material was suspended in PBS at an approximate 1:10 dilution, clarified by centrifugation at 3000 rpm for 30 minutes, and the supernatant heated to 56°C for 30 minutes. The supernatant was then allowed to equilibrate to room temperature before being subjected to the HAI test.

Preparation of pig erythrocyte suspension. The 1% suspension of pig erythrocytes was prepared as described for the haemagglutination test. The 50% suspension was prepared by resuspending sedimented erythrocytes in an equal volume of PBS.

Preparation of antigen. CPV was grown in tissue culture by inoculating pre-confluent monolayers of FEA cells with stock CPV, isolated originally from the faeces of a dog with CPV enteritis (McCandlish *et al.*, 1979). Cultures were first prepared by inoculating 8 oz. flat sided bottles with 2×10^6 FEA cells suspended in Eagle's medium with 10% FBS. The cultures were incubated at 37°C for two hours to permit the cells to attach to the glass. The growth medium was then removed and 1 ml of stock virus suspension added to the culture. Following a further two hours' incubation at 37°C, 20 ml of Eagle's medium with 10% FBS was added and the culture incubated at 37°C for 4 days, when both cells and fluid were harvested. The harvested culture was rapidly frozen and thawed three times, clarified by centrifugation, assayed for haemagglutinin titre and stored at -20°C until required.

PBS

This was prepared by dissolving one Dulbecco A PBS tablet (Oxoid, Basingstoke) in 100 ml of water which had been distilled in a glass distillation unit.

Post-mortem Examination

All dogs were subjected to full post-mortem examination as soon after death as possible. A few dogs died during the course of the experiments but most were killed by overdosing with pentobarbitone sodium (Euthatal, May & Baker Ltd., Dagenham) by the intravenous route.

All organs were examined and tissues were taken for histological, immunocytochemical and electron microscopical examination.

Histological Procedures

In all experiments, tissues were fixed in 10% Neutral Buffered Formalin (NBF) and, in Chapters 6 and 7, tissues were also fixed in Bouin's fixative. Small portions of the following tissues were taken and placed in the appropriate fixatives:

Sub-mandibular, retropharyngeal, mesenteric and popliteal lymph nodes, thymus, palatine tonsil, spleen, lung, heart, liver, kidney, bone marrow, stomach, duodenum, jejunum, ileum, caecum and colon. With the exception of the stomach and caecum, the alimentary tissues were pinned flat on cork blocks before fixation.

At post-mortem examination, the entire small intestine was removed and straightened out along its length. Samples of duodenum were taken 1-2 cm distal to the pancreatic papilla, samples of ileum were taken 5-6 cm proximal to the ileocaecocolic valve and jejunal specimens removed at a point equidistant between the pylorus and caecum.

Fixation in 10% NBF. Tissue blocks remained in fixative for a minimum of 24 hours, when each tissue was trimmed to a thickness of not more than 4 mm, with a flat surface provided to ensure accurate orientation and to facilitate sectioning. After

trimming, all specimens were transferred to fresh 10% NBF, and duplicate portions post-fixed in corrosive formal for a further 24 hours. All blocks were then processed as detailed below.

Fixation in Bouin's Fixative. Blocks no thicker than 3 mm were placed in this fixative for between 18 and 24 hours. After trimming, the blocks were transferred to 70% ethanol for 24 hours and processed.

Fixatives were prepared as follows:

10% Neutral Buffered Formalin (NBF)

Tap water	900 ml
Concentrated formaldehyde (40%)	100 ml
Sodium dihydrogen orthophosphate	4.6 g/l
Dipotassium hydrogen orthophosphate	8.0 g/l

Corrosive Formal (Formal sublimate)

Concentrated Formaldehyde (40%)	100 ml
Mercuric chloride (sat. aqueous soln.)	900 ml

Bouin's Fixative

Concentrated Formaldehyde (40%)	250 ml
Picric acid (sat aqueous soln.)	750 ml
Glacial acetic acid	50 ml

Processing

Tissues were processed by dehydration through a series of alcohols, double embedded in 1% celloidin in methyl benzoate and embedded in paraffin wax, using amyl acetate as antemedia. Sections were cut at a thickness of 3 μ and stained using Meyer's Haematoxylin and Eosin.

Immunocytochemical Procedures

The development of both immunofluorescence and immunoperoxidase methods of immunocytochemistry is described in detail in Chapter 5. Selected blocks from NBF fixed, corrosive formal post-fixed tissues were examined by the immunoperoxidase method while the full range of tissues listed under histological procedures was examined by immunofluorescence. For the latter, duplicate blocks were snap frozen by immersion in liquid nitrogen and sectioned on a Slee cryostat at a thickness of 6 μ . The tissue blocks were then stored immersed in liquid nitrogen in airtight capsules for further study.

Ultrastructural Procedures

A. Scanning Electron Microscopy (SEM)

In Chapter 7, portions of duodenum, jejunum, ileum and colon were examined by SEM. Small pieces of these tissues, not more than 16 mm² were removed as soon after death as possible. The tissues were fixed in chilled glutaraldehyde/paraformaldehyde fixative for 4 hours. The tissue was then trimmed into blocks with a surface area not greater than 4 mm² and washed overnight in cacodylate buffer. Tissue blocks were dehydrated through an ascending series of acetones, with a final overnight dehydration in 100% acetone after which they were dried in a Polaron Critical Point Drier (Polaron, Watford). Tissues were mounted on a Philips standard 501B SEM stub, using conductive silver paint (Agar Aids, Stanstead), the specimens were gold-coated using a Sputter coater (Emscope, London) and examined using a Philips 501B scanning electron microscope.

B. Transmission Electron Microscopy (TEM)

TEM was used to examine tissue used in the development of the immunocytochemical techniques described in Chapter 5.

Small pieces of heart muscle were removed as soon after death as possible, and placed in drops of chilled paraformaldehyde/Glutaraldehyde fixative on blocks of dental wax. These small blocks were then transferred to glass phials containing chilled fixative and were fixed at 4°C for four hours. Following fixation, each tissue was rinsed overnight in cacodylate rinsing solution and post-fixed in osmium tetroxide for one hour.

The fixed tissue was dehydrated through an ascending series of 70%, 90% and absolute alcohol, and was rinsed in propylene oxide. The tissues were soaked for one hour in a mixture of equal parts propylene oxide and araldite, and left overnight in an 80% araldite mixture. Individual blocks were then embedded and the embedding resin polymerised at 57°C for 48 hours.

Sections 1 μ thick were cut on a LKB Mark III ultramicrotome using glass knives, mounted on glass slides and stained with toluidine blue (Trump et al., 1961). Fields for ultramicroscopy were then selected and the original blocks trimmed accordingly.

Ultrathin sections were cut on the ultramicrotome and mounted on uncoated Athene 482 copper specimen grids (Agar Aids, Stanstead). Sections were stained with Uranyl acetate, rinsed in methanol, 50% methyl alcohol in distilled water, after which the grids were dried on filter paper. They were then stained for 10 minutes with lead citrate, rinsed with 0.02N sodium hydroxide, distilled water and again air dried on filter paper.

Sections were examined using an AEI 6B electron microscope.

Paraformaldehyde/Glutaraldehyde mixture:

1.3% paraformaldehyde and 1.6% glutaraldehyde in cacodylate buffer pH 7.2 - 7.4.

Paraformaldehyde	2g
Distilled water	25 ml
1M sodium hydroxide	2-3 drops
25% glutaraldehyde	10 ml
Cacodylate buffer	115 ml
Anhydrous calcium chloride	25 mg

Cacodylate Buffer: This was prepared as a 0.1M solution of sodium cacodylate in distilled water (21.4g/l) and adjusted to pH 7.4 - 7.6 by the addition of a few drops of concentrated hydrochloric acid.

Cacodylate Rinsing Solution: This was prepared by dissolving sucrose in cacodylate buffer, 34.2g sucrose/litre, resulting in a 0.1M solution of sucrose. The pH was again adjusted to lie between 7.2 and 7.4 by the addition of a few drops of concentrated hydrochloric acid.

Osmium tetroxide: 1% osmic acid (BDH Chemicals Ltd., Poole, Dorset) in Millonig's buffer pH 7.2 - 7.4.

Millonig's phosphate buffer was prepared as follows:-

Sodium dihydrogen phosphate (2.26%)	83 ml
Sodium hydroxide (2.52%)	17 ml
Distilled water	10 ml
Sucrose	0.54 g.

Uranyl Acetate: A 20% solution (May and Baker, Dagenham) was made up in 100% methanol.

Lead Citrate: Lead nitrate (1.33g) and sodium citrate (1.75g)

were dissolved in separate 15 ml volumes of distilled water. The solutions were then mixed, the lead citrate precipitate shaken for one minute and then allowed to stand for 30 minutes with periodic agitation. The precipitate was solubilised by the addition of 8 ml of 1M sodium hydroxide. The solution was diluted to 50 ml with distilled water. The final pH was 11.9 - 12.1.

Araldite mixture: Equal parts Araldite Resin (CY 212) and Araldite Hardener (HY 964) were mixed by stirring overnight and then stored at 4°C until required. Before use in embedding, 0.6 ml of accelerator (DH 064) and 2.4 ml of di-n-butyl phthalate were added to 57 ml of the araldite mixture, and the total volume stirred for 30 minutes.

CHAPTER 3 : PRELIMINARY INVESTIGATION

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INTRODUCTION

The aim of this experiment was to evaluate the response of susceptible dogs to oral infection with CPV of tissue culture origin. In addition, this experiment was to provide a baseline for subsequent, more detailed investigations of the pathogenesis.

MATERIALS AND METHODS

Experimental Animals

The source, housing and maintenance of the experimental animals has been described (Chapter 2). In this experiment, 10, 12-week old beagle pups were used.

Experimental Design

The 10 puppies were randomly divided into three groups upon their arrival at the laboratory (Table 1). Two puppies were killed at the start of the experiment to monitor the health status of the dogs and to obtain uninfected control tissue. Two puppies were kept as in-contact, non-infected controls and the remaining six puppies were each orally infected with CPV. The dogs were infected at 10 a.m., having been fed the previous night at 5 p.m. The dogs were subsequently offered food two hours after infection. Each dog received 2 ml of virus suspension in its 6th passage in tissue culture. The suspension had an HA titre of 256 per 0.025 ml and the infectivity titre was $6.0 \log_{10} \text{TCID}_{50}$ per ml. The method of preparation was detailed in Chapter 2. The infected and in-contact dogs were subsequently housed in a communal pen.

All dogs were examined clinically each day and rectal swabs were taken for the detection of virus excretion. Each day, blood samples were removed by ear vein laceration for haematological examination and 5 ml of blood was collected by

jugular venipuncture for serological examination on the day of infection and on the day of death.

Infected animals were killed at 2, 3, 4, 6, 9 and 12 days post-infection (DPI). The contact controls were killed at days 10 and 12 after infection of the other dogs (Table 1). A full post-mortem examination was performed on all animals and tissues taken for histological examination into 10% NBF as detailed in Chapter 2.

Procedures Employed

Clinical and post-mortem examinations, haematological, serological and histological procedures and detection of virus excretion were carried out as detailed in Chapter 2.

RESULTS

Control Dogs

The control dogs killed at the start of the experiment were clinically healthy. Rectal temperatures were within the normal range. The values obtained from haematological examination are recorded in Tables 4, 5 and 6. Viral haemagglutinins were not detected in the rectal swabs from these animals and antibody to CPV was not detected in the serum samples. Post-mortem examination revealed no macroscopic abnormalities and histological examination revealed no underlying pathological lesions.

Infected and In-contact Dogs

Clinical Signs

Clinical signs in the infected dogs were variable in both their appearance and their severity (Table 2). Dog 3, killed at 2 DPI remained clinically normal and dullness and anorexia were first observed in dog 4, at 3 DPI. Dog 5 was also dull and anorexic on the day it was killed (4 DPI). At 5 DPI, dogs 6, 7 and 8 were dull; dogs 6 and 8 became progressively duller, and were completely anorexic by days 6 and 7 respectively. Dog 7 was still slightly dull at 6 DPI, although its condition had not deteriorated from that of 5 DPI. Dogs 7 and 8 then showed a progressive recovery, becoming brighter by 8 and 9 DPI respectively. Abnormal faeces were not consistently observed and diarrhoea was noted in only one of the infected dogs, dog 6 at 6 DPI. The faeces were softer than normal at this time and appeared to contain excess amounts of mucus. Blood was not observed in the faeces of any infected animal. A further, and more consistent sign, was enlargement of palpable peripheral lymph nodes in all infected dogs, first noted at 3 and 4 DPI and present in all dogs after this time.

Of the two in-contact dogs, only dog 9 showed clinical signs of illness, while dog 10 remained clinically normal, apart from some enlargement of peripheral lymph nodes, first observed at 7 DPI. In dog 9, progressive dullness and inappetance was noted from 8 DPI until, by day 10, there was marked depression (Fig. 1). Diarrhoea was first noted on day 9 and on the morning of day 10 there was frequent vomition and profuse watery diarrhoea. By the afternoon of day 10, the animal was passing dysenteric faeces (Fig. 2), had become severely dehydrated and rapidly became prostrate. The dog was killed in extremis that afternoon.

The daily rectal temperatures of the dogs are recorded in Table 3. There was some variation in the rectal temperatures on the day of infection and during the course of the experiment but nonetheless, there did appear to be a rise in temperature during the period of clinical illness, especially on day 5 when dogs 6 and 8 had rectal temperatures above 39.5°C, the accepted upper limit of normality in the dog. An elevated rectal temperature was also recorded in dog 9, a contact control, on the first and second days of clinical illness.

Haematological Findings

The haematological findings are recorded in Tables 4, 5 and 6. There was a wide variation in the total white cell counts on the day of infection with values ranging from 5.76 to 23.05 x 10⁹/litre. In the course of the experiment, a marked rise in total leukocyte counts with an absolute neutrophilia was recorded in all of the dogs. In one dog (dog 6) a total white cell count of 54.85 x 10⁹/litre with 90% neutrophils was recorded on the 5th day after infection.

Although the technique of ear vein laceration was well tolerated by the dogs, technical problems in collecting blood samples by this route were evident. There was variable clotting of the sample and localised thickening and inflammation along the line of the auricular vein made collection increasingly difficult as time progressed.

Viral Excretion

Viral specific haemagglutinins were detected at 1 DPI in four of the six infected dogs (Table 7) and were still present in one dog (dog 3) at 2 DPI. Viral haemagglutinins were not detected again in the infected dogs killed on days 3 and 4. In dog 6, killed on day 6, levels of 32 were recorded in swabs collected on days 5 and 6. Somewhat surprisingly, haemagglutinins were not detected at all in dog 7 after the

initial low level of day 1. Virus was detected in the swabs from dog 8 on days 5 and 7, the level on day 7 being massive. Viral haemagglutinins were detected in the swabs from both of the in-contact dogs. Virus was first detected in dog 10 at 6 DPI but was not recorded from dog 9 until 10 DPI.

Serological Findings

The serological findings are shown in Table 8. Specific antibodies to CPV were not detected in any of the dogs on the day of infection. Antibody was first noted in dog 5, killed at 4 DPI when a level of 4 was recorded. In dog 6, killed at 6 DPI, the antibody titre was significantly higher, at 512. In dogs sampled after this time, levels greater than or equal to this were found. Levels of 2048 and 4096 were recorded in the contact control dogs.

Pathological Findings

Macroscopical Findings

The findings at post-mortem examination are summarised in Table 9. No abnormalities were detected in dog 3, killed at 2 DPI. In the dogs killed at 3 and 4 DPI the lymph nodes, both carcase and visceral, were enlarged and appeared oedematous on cut section; in dog 5, killed at 4 DPI, the thymus was also swollen and oedematous.

The first dog to be examined while it was clinically ill, dog 6, killed at 6 DPI, had changes present in the intestinal tract, the lymph nodes and the thymus. The lymph nodes were again enlarged; in general, oedema was less marked, the cortices being prominent and bulging but in the mesenteric and ileocaecocolic nodes which were strikingly enlarged, oedema was still present. The thymus was smaller than expected and petechiae were present on the serosal and cut surfaces; petechiation was particularly marked in the anterior lobe. In

the alimentary tract, the wall of the small intestine was thickened and the mucosa appeared generally oedematous. Congestion was noted only in the ileum where transverse bands of congestion were present on the mucosal aspect, whereas the serosal surface was more diffusely reddened. The stomach, caecum and colon were unremarkable.

In dogs 7 and 8, killed at 9 and 12 DPI, after they had made a clinical recovery, changes were confined, once again, to the lymph nodes and thymus. The nodes were hyperplastic. The thymus in dog 7 was reduced to approximately half the volume found in the control dogs; in dog 8, there was almost complete atrophy of this organ which appeared to be reduced effectively to its connective tissue elements (Fig. 3).

In both contact controls, dogs 9 and 10, killed at 10 and 12 DPI, similar changes were present in the lymph nodes and thymus to those found in infected animals. The lymph nodes were hyperplastic and the thymus was reduced in size with some petechiation evident in the thymus of dog 9; also in dog 9, the mesenteric and ileocaecocolic nodes were greatly enlarged and oedematous. As in the infected group, changes in the alimentary tract were confined to the dog clinically ill at the time of death; this was dog 9, which had been killed in extremis. In this animal, the wall of the small intestine was diffusely thickened and the serosal surface had a finely granular appearance (Fig. 4) not unlike the characteristic surface of Early English salt glazed pottery.

Microscopical Findings

The microscopical findings are summarised in Table 10. No abnormalities were noted in dog 3, the infected dog killed at 2 DPI. In dog 4, killed at 3 DPI, early lymphocytolysis was noted in lymphoid tissues throughout the body. In the lymph nodes and gut associated lymphoid tissue (GALT), numerous cells with pyknotic nuclei were evident in the

germinal centres; similar cells were present in the splenic white pulp and in the thymic cortex. In the thymus, changes were more advanced with some fragmentation of thymocytes as well as nuclear pyknosis. (Fig. 5).

The lesions in dog 5, killed at 4 DPI, were similar in distribution but were more severe, with many more fragmented lymphocytes (Fig. 6), particularly in the germinal centres and thymic cortex. Pale, amphophilic intranuclear inclusion bodies were present in some of the non-fragmented centrocytes of the lymph nodes and palatine tonsil. In addition, there was extensive interlobular oedema in the thymus.

In dog 6, killed at 6 DPI, severe and extensive lesions were present throughout the lymphoid tissues and histological abnormalities were also present in the intestinal tract. In the thymus, there was advanced lymphocytolysis with cortical depletion (Fig. 7); interlobular oedema was still evident but was less of a feature than in dog 5. Active lymphocytolysis was less pronounced in the lymph nodes, tonsils and GALT. These tissues were depleted of lymphocytes, particularly in the germinal centres which were now composed predominantly of large "histiocytic" cells (Fig. 8) with abundant eosinophilic cytoplasm and round or ovoid nuclei with a stippled chromatin pattern. In the GALT, some of the germinal centres were, in fact, necrotic and composed of amorphous debris (Fig. 9); occasional amphophilic intranuclear inclusion bodies were visible in the centrocytes of the non-necrotic centres (Fig. 10) and active lymphocytolysis was still evident in the perifollicular lymphoid tissue of the GALT.

In the intestines of this dog, lesions were found in the duodenum, jejunum and ileum. The duodenal villi were short and the apical epithelial cells vacuolated. There were numerous small darkly amphophilic staining cells in which the nucleus and cytoplasm appeared to be contracted and could not easily be differentiated (Fig. 11). In the proliferative zone

of the crypt epithelium, some of these cells were detached from the basement membrane and lay free in the crypt lumen. In other crypts, there appeared to have been a loss of cells and the remaining lining cells were attenuated; the lumen of these crypts appeared dilated. Similar changes were observed in the jejunum: although here the small amphophilic cells were more numerous and there were fewer dilated crypts with a flattened epithelium.

In the ileum, severe changes similar in nature to those described in the duodenum and jejunum were noted in the areas immediately overlying the GALT. In other areas of the ileum, changes were very slight. No epithelial lesions were noted in the stomach, caecum or colon.

In dogs 7 and 8, killed at 9 and 12 DPI, lesions were again detected only in the lymphoid tissues. In the thymus, no active cellular destruction was seen but there was severe depletion of cortical lymphocytes, particularly in dog 8. In the lymph nodes, there was evidence of regeneration with repopulation of the germinal centres by centroblasts and centrocytes (Fig. 12); there was also marked expansion of the paracortex and an increase in numbers of plasma cells in the medullary cords. In the spleen, the white pulp was similarly expanded and germinal follicles were prominent. Lesions in the intestinal tract were limited to the GALT where again there was evidence of regeneration and lymphoid expansion.

In in-contact dog 9, which had been killed in extremis, there was active lymphocytolysis in the germinal centres of lymph nodes and splenic white pulp; similar lesions were present in the GALT. There was severe depletion of cortical thymocytes and although no active lymphocytolysis was noted there was an infiltrate of neutrophils in the thymic cortex. Extensive intestinal lesions were present in this animal. In the duodenum, there was stunting of some villi with dilation of the related crypts. The latter were again lined by

an attenuated epithelium and a few amphophilic intranuclear inclusion bodies were noted in some of these cells (Fig. 13). Lesions were more extensive and severe in the jejunum. Here there was virtually complete collapse of the mucosa with total loss of villi and the crypts were discernible in the mucosa only as dilated spaces lined by attenuated epithelial cells and filled with necrotic debris (Fig. 14). In many areas, the surface epithelium had been lost and the remaining surface epithelial cells were flattened or cuboidal with foamy or vacuolated cytoplasm. The mucosa was generally congested and mild infiltration by inflammatory cells was a feature. Severe lesions similar to those in the jejunum were present in the ileum in areas overlying and adjacent to the GALT; in other areas, changes were more focal and less severe.

In dog 10, the in-contact killed at day 12, histological changes consisted of cortical depletion of the thymus, reactive hyperplasia of the lymph nodes and expansion of the GALT.

DISCUSSION

The results of this experiment demonstrated that disease could be produced by oral infection of susceptible dogs with tissue culture derived CPV; and that infection and disease could develop in in-contact dogs.

It would have been preferable to maintain uninfected control dogs throughout this experiment to provide a baseline for the evaluation of clinical, haematological and virological parameters. However, it was not possible to maintain these control dogs in isolation due to a shortage of accommodation and consequently keeping them free from infection would have presented major problems. The animals were therefore killed at the start of the experiment to provide non-infected tissues and to monitor the health status of the group.

The clinical syndrome produced following infection was variable in severity although all of the infected dogs which were kept more than 2 DPI showed at least some degree of dullness and anorexia. These signs were most marked around 6DPI and at this time one of the infected dogs did develop diarrhoea. The variability of clinical disease was even more striking in the in-contact animals. Both in-contacts became infected, as evinced by the high antibody titres to CPV which they developed; but one animal showed no clinical signs of illness while the other developed a severe syndrome identical to that seen in the naturally-occurring disease.

This variable clinical pattern of disease is similar to that found in field outbreaks where, in infected litters, only a proportion of pups may die while others show clinical illness of varying severity and some remain apparently normal (Harcourt et al., 1980; McCandlish et al., 1981). This variability has also been observed by other workers following experimental challenge. (Carmichael et al., 1981; Potgeiter et al., 1981; Pollock, 1982).

The clinical variability was reflected in the range and severity of macroscopic and microscopic changes in the intestinal tracts of infected and in-contact dogs. Changes were found in the intestinal epithelia only in the two dogs which were clinically affected when killed. There was a correlation between the extent of the intestinal lesions and the severity of the clinical signs, with more extensive lesions in the more severely ill in-contact animal.

In contrast, pathological changes were consistently present in the lymphoid tissues of all infected and in-contact animals (with the exception of the dog killed at 2 DPI). The earliest changes were destructive, with lymphocytolysis and pyknosis which was evident in lymph nodes, spleen, GALT and thymus. This resulted in a generalised lymphoid depletion, most marked in germinal centres, followed by repopulation of the tissues with reactive hyperplasia in the nodes, spleen and GALT. In the thymus, destruction of mature lymphoid cells was particularly evident and resulted in obvious atrophy of this organ and which was still apparent in the infected and in-contact dogs killed at 12 DPI. As this thymic change was important and consistent, it was felt that it should be quantified and it was therefore decided that in future experiments the thymus should be weighed and related to the total body weight of the dog.

Although extensive destructive lesions were present in the lymphoid tissues, only occasional intranuclear inclusions indicative of the presence of virus were found. Similarly, in intestinal epithelial cells distinct inclusion bodies were only rarely seen and were found only in the two dogs with obvious intestinal lesions. The small dark amphophilic cells found in the intestinal epithelia of these dogs with intestinal lesions may also have been a form of viral inclusion but it was impossible to be certain of this solely on histological examination. Moreover, although there were no intestinal epithelial lesions recognisable in the other infected dogs,

without a specific method of visualising the presence of viral antigen, one could not eliminate the possibility of viral localisation without overt lesions in the intestinal epithelia at other times.

It was apparent that in order to visualise the distribution of viral antigen throughout the tissues, and to relate this accurately to the pathogenesis of the lesions, particularly early in the course of the disease and especially in the intestinal tract, immunocytochemical techniques would need to be applied. The development of immunocytochemical techniques and their application to CPV infection is described in Chapter 5. In addition, Carlson and coworkers (1977a) and Shindel and his colleagues (1978) have remarked that the inclusions in feline parvovirus are more easily discerned following fixation in Bouin's fixative (due to the acidic nature of this fixative) and so it was also decided that in future studies duplicate tissue blocks would be taken into this solution.

In the course of this preliminary study, inadequacies were immediately apparent in certain aspects of the experimental design.

First, the method of daily collection of blood samples by ear vein laceration was a mistake. This route had been chosen as offering a well-tolerated, simple and quick method of repeated collection of blood in small and highly mobile puppies. In practice, although certainly well tolerated by the pups, the method was not rapid because of problems with clotting of the sample as it was collected and with control of bleeding from the vein after collection. Moreover, the results of haematological examination on these samples were unexpected with both wide variations in initial values and increases in the absolute neutrophil counts in most dogs. These results are in marked contrast both to standard values for the dog (Schalm et al., 1975) and the results obtained by other workers with both

CPV (Appel et al., 1979a) and FPV (Larsen et al., 1976). Sampling from a peripheral site such as the ear vein might be expected to give a wider variation than would sampling from a more central site; factors such as local blood flow, local sequestration of leukocytes and microclotting during collection could all have contributed to the range of values obtained. Moreover, there was undoubtedly, as the experiment progressed, considerable local inflammation and reaction along the ear which must have contributed to the high neutrophil counts found. The ear vein technique was therefore considered unsatisfactory, giving unreliable and unrepresentative results. It was decided that in future experiments blood samples would be obtained by jugular venipuncture.

Second, the method of detection of viral excretion by examination of rectal swabs was to some extent unsatisfactory. Rectal swabs were collected because the communal housing of the animals, used to permit the inclusion of contact controls, made the individual identification of faecal specimens from specific dogs impossible. The problem with the rectal swabs was that, in some instances, the amount of faecal material on the swab was minimal. As the HA test is a quantitative one, this lack of material could have led to some false negative results or to an underestimation of the amount of haemagglutinin in rectal faeces, as opposed to that on the surface of the rectal mucosa. It was decided that in future experiments infected dogs should be housed individually. This would not only permit the collection of individual faecal specimens to optimise the detection of viral excretion but would also allow more accurate monitoring of individual food and water intake.

Allowing for the limitations of the collection technique mentioned above, examination of the rectal swabs revealed a biphasic pattern of virus excretion. There was excretion of virus in all but one of the infected dogs at 1 DPI, which must have represented passive shedding of the oral challenge. Virus was next detected in the faeces of infected

dogs at 5 DPI, coinciding with the onset of dullness and anorexia; this virus excretion was presumably the result of virus replication in the intestine.

It is interesting and possibly significant that the first detection of viral excretion from the in-contact dogs was in dog 10 at 6 DPI although virus was not detected in the other in-contact, dog 9, until 10 DPI. If one applies the time scale of viral excretion from the infected dogs to the contact controls (namely that active excretion first occurs 5 days after infection), then it is possible that dog 10 acquired infection from the passively excreted challenge virus of 1 DPI whereas dog 9 was infected by the actively excreted virus released at 5 DPI. If this was indeed the case then it could be significant that none of the dogs infected with tissue culture-derived virus, i.e. the infected dogs and contact dog 10, developed severe clinical disease, whereas dog 9, the contact infected by actively excreted "dog-passaged" virus developed a severe illness akin to the naturally-occurring disease. The possible explanations for this observation could be either that passage in tissue culture in some way results in attenuation of the virus, or that active multiplication of CPV in infected animals results in a much larger challenge to in-contact dogs.

Since the nature of the challenge could be important it seemed logical to try to improve the quality and quantity of the challenge material. The steps taken in pursuit of this aim are described in the following section (Chapter 4).

TABLE 1.

PRELIMINARY INVESTIGATION: EXPERIMENTAL DESIGN

Dog number		Day killed	
Group 1	1	0	Control Group
	2	0	
Group 2	3	2	Infected Group
	4	3	
	5	4	
	6	6	
	7	9	
	8	12	
Group 3	9	10	Contact Control Group
	10	12	

TABLE 2

PRELIMINARY INVESTIGATION: CLINICAL SIGNS

Dog No.	0	1	2	3	4	5	6	7	8	9	10	11	12
1	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	A+	-	-	-	-	-	-	-	-	-
5	-	-	-	-	A+	-	-	-	-	-	-	-	-
6	-	-	-	-	-	A+	A+++	D+	-	-	-	-	-
7	-	-	-	-	-	A+	A+	A+	-	-	-	-	-
8	-	-	-	-	-	A+	A++	A+++	A+	-	-	-	-
9	-	-	-	-	-	-	-	-	A+	A++	V A+++	-	-
10	-	-	-	-	-	-	-	-	-	D++	D+++	-	-

A = Dullness and Anorexia, + to +++ on severity. V = Vomition

D = Diarrhoea + = Mucoïd

++ = Fluid

+++ = Dysenteric

TABLE 3

PRELIMINARY INVESTIGATION: RECTAL TEMPERATURES (°C)

Dog No.	Day Post Infection												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1	39.1												
2	39.3												
3	39.1	38.8	38.5										
4	38.3	38.6	38.6										
5	38.2	38.5	38.9	38.9	39.3								
6	38.4	38.5	38.5	38.3	38.9	39.7	38.9						
7	38.8	39.1	39.1	38.8	39.8	39.1	38.8	38.4	38.7	38.4			
8	38.6	38.4	38.8	38.5	39.1	39.9	38.8	38.4	38.3	38.8	38.5	38.3	38.8
9	38.1	38.2	38.6	38.7	37.9	38.4	38.4	37.9	39.6	40.1	39.3		
10	39.2	38.6	38.8	38.3	38.9	38.8	39.1	38.7	38.8	38.6	38.6	38.6	38.6

TABLE 4

PRELIMINARY INVESTIGATION: TOTAL CIRCULATING LEUKOCYTE COUNTS ($\times 10^9/\text{litre}$)

Dog No.	Day Post Infection												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1	9.993												
2	9.975												
3	20.73	35.94	15.99										
4	20.12	14.77	10.96	15.1									
5	18.04	23.83	21.94	25.96	31.68								
6	23.05	32.21	23.96	31.02	33.56	54.85	16.28						
7	5.76	15.54	15.15	16.91	29.99	22.51	12.01	20.98	16.25	17.83			
8	21.5	29.53	26.76	28.66	29.03	29.2	17.06	21.8	18.84	17.21	15.89	35.92	25.11
9	11.92	19.72	20.07	24.83	26.27	33.27	19.22	12.68	24.34	12.07	6.41		
10	9.55	10.67	18.39	21.73	23.12	17.29	8.7	11.12	12.71	14.77	10.84	14.32	20.06

TABLE 5

PRELIMINARY INVESTIGATION: ABSOLUTE CIRCULATING NEUTROPHIL COUNTS ($\times 10^9/\text{litre}$)

Dog No.	Day Post Infection												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1	6.59												
2	7.48												
3	13.05	24.08	10.63										
4	14.28	9.89	13.22	9.89									
5	12.81	17.39	15.91	16.1	25.66								
6	17.28	20.13	18.21	22.02	26.01	48.26	12.45						
7	3.4	11.03	9.69	10.99	25.19	18.23	6.72	12.9	7.47	12.30			
8	14.83	21.26	20.73	16.62	22.5	25.25	12.96	14.83	11.11	9.12	8.1	21.37	14.31
9	8.34	13.80	14.01	15.64	16.28	17.29	11.53	8.05	21.42	9.3	4.16		
10	5.44	7.47	12.69	13.47	16.07	12.44	6.96	6.11	7.11	10.04	7.53	8.02	13.69

TABLE 6

PRELIMINARY INVESTIGATION: ABSOLUTE CIRCULATING LYMPHOCYTE COUNTS ($\times 10^9/\text{litre}$)

Dog No.	Days Post Infection												
	0	1	2	3	4	5	6	7	8	9	10	11	12
1	3.09												
2	2.09												
3	7.46	10.42	4.95										
4	5.63	4.13	3.39	4.3									
5	5.05	5.95	5.48	8.82	5.54								
6	5.3	10.79	4.01	8.06	4.69	5.48							
7	2.24	3.88	5.15	5.58	3.14	3.15	4.56	7.03	7.07	4.72			
8	5.8	6.79	4.54	10.6	4.2	2.22	2.73	5.23	6.5	6.36	6.19	12.21	5.27
9	3.33	5.12	4.9	7.07	8.8	13.64	6.53	3.55	2.19	1.55	0.83		
10	4.01	3.09	5.51	8.04	6.7	4.32	1.56	4.28	3.49	4.06	2.21	4.58	5.66

TABLE 7

PRELIMINARY INVESTIGATION: EXAMINATION OF FAECAL SWAB SUPERNATANTS
FOR THE PRESENCE OF SPECIFIC CPV HAEMAGGLUTININ*

Dog No.	Day Post Infection											
	0	1	2	3	4	5	6	7	8	9	10	11 12
1	< 2											
2	< 2											
3	< 2	4	2									
4	< 2	< 2	< 2	< 2								
5	< 2	< 2	< 2	< 2	< 2							
6	< 2	4	< 2	< 2	< 2	32						
7	< 2	4	< 2	< 2	< 2	< 2	< 2	< 2	< 2	2		
8	< 2	8	< 2	< 2	< 2	8	< 2	4096	< 2	2	< 2	< 2
9	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	< 2	2	16	
10	< 2	< 2	< 2	< 2	< 2	< 2	16	4	< 2	2	< 2	< 2

* Results are expressed as the reciprocal of the highest dilution giving complete agglutination.

TABLE 8

PRELIMINARY INVESTIGATION: EXAMINATION OF SERA FOR THE PRESENCE
OF HAEMAGGLUTINATION INHIBITING ANTIBODY TO CPV*

Dog No.	0	1	2	3	4	5	6	7	8	9	10	11	12
1	< 4												
2	< 4												
3	< 4	-	< 4										
4	< 4	-	-	< 4									
5	< 4	-	-	-	4								
6	< 4	-	-	-	-	-	512						
7	< 4	-	-	-	-	-	-	-	-	2048			
8	< 4	-	-	-	-	-	-	-	-	-	-	-	512
9	< 4	-	-	-	-	-	-	-	-	-	2048		
10	< 4	-	-	-	-	-	-	-	-	-	-	-	4096

* Results are expressed as the reciprocals of the highest dilutions which completely inhibited agglutination.

TABLE 9

PRELIMINARY INVESTIGATION: POST-MORTEM FINDINGS

Dog No.	Day Killed	Macroscopic Findings
1	0	NAD
2	0	NAD
3	2	NAD
4	3	Enlarged, oedematous lymph nodes.
5	4	Enlarged, oedematous lymph nodes, swollen oedematous thymus.
6	6	Enlarged lymph nodes with bulging cortices. Thymus reduced in size and petechiated. Small intestine thickened, congestion of ileum.
7	9	Enlarged lymph nodes with bulging cortices. Thymus markedly reduced in size with petechiae.
8	12	Enlarged lymph nodes with bulging cortices. The thymus was markedly reduced in size.
9	10	Enlarged lymph nodes with bulging cortices. Thymus reduced in size with petechiae. Small intestine thickened and congested, serosal surface granular.
10	12	Enlarged lymph nodes with bulging cortices. Thymus reduced in size.

NAD = No abnormalities detected.

TABLE 10

PRELIMINARY INVESTIGATION: MICROSCOPIC FINDINGS

Dog No. Day killed		Microscopic Findings
1	0	NAD
2	0	NAD
3	2	NAD
4	3	Pyknotic cells in lymph nodes and GALT. Pyknosis and fragmentation cortical thymocytes.
5	4	Fragmentation of lymphoid cells in thymic cortex and germinal centres of lymph nodes. Ampophilic intranuclear inclusion bodies in lymph nodes and tonsil.
6	6	Depletion of germinal centres and thymus. Germinal centres replaced by histiocytic-like cells. Short intestinal villi, some small dark ampophilic cells in crypt epithelium with some attenuation of epithelium.
7	9	Depletion of cortical thymocytes. Repopulation of germinal centres. Paracortical expansion in the lymph nodes.
8	12	Depletion of cortical thymocytes. Repopulation of germinal centres with paracortical expansion in the lymph nodes.
9	10	Depletion of thymic cortex, with depletion and some residual lymphocytolysis in the germinal centres. Loss of villi with dilation of crypts and attenuation of lining epithelium in the duodenum, and more extensively in the jejunum.
10	12	Depletion of thymic cortex, reactive hyperplasia of lymph nodes, and expansion of the GALT.

NAD = No abnormalities detected.

Fig. 1: Canine Parvovirus Infection - Dogs 8, 9, 10.

Note the marked depression in Dog 9 (arrowed), when compared to the demeanor of Dogs 8 and 10, taken at 10 DPI.

Fig. 2: Canine Parvovirus Infection - Faeces of Dog 9.

The faeces are mucoid and haemorrhagic, taken at 10 DPI.



Fig. 3: Canine Parvovirus Infection - Thymus of Dog 8.

The thymus (arrowed) is reduced to its connective tissue elements. Taken at 12 DPI.

Fig. 4: Canine Parvovirus Infection - Intestine of Dog 9.

The serosal surface of the small intestine is finely granular in appearance. The wall of the intestine is thickened, the contents are scant and are fluid in consistency. Contact control dog, taken at day 10.

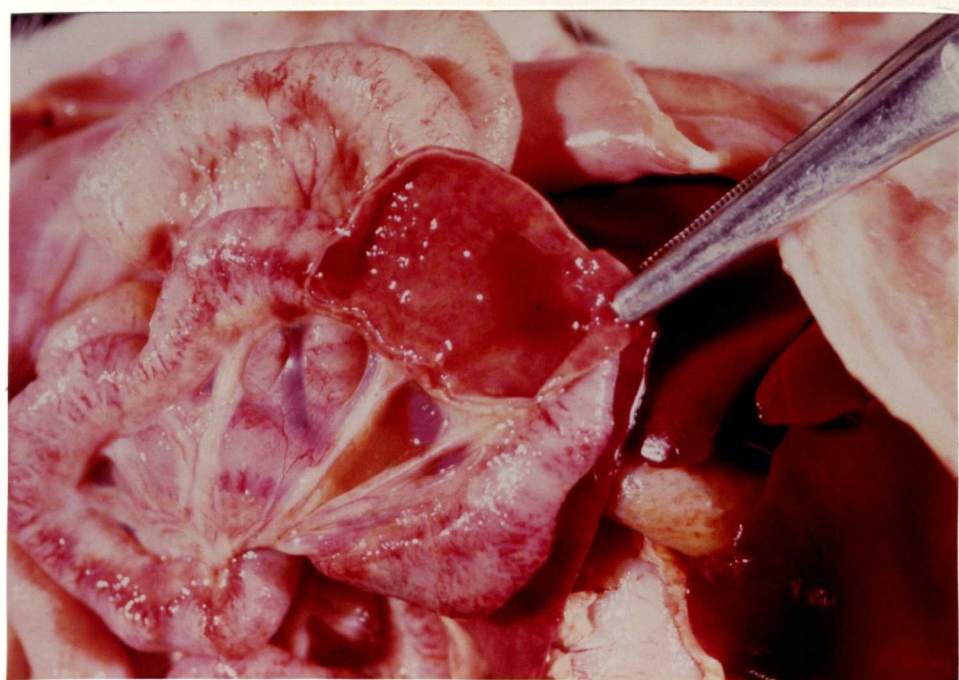


Fig. 5: Canine Parvovirus Infection - Thymus of Dog 4.

The thymic cortex appears irregular, rather than uniformly cellular, because of pyknosis and fragmentation of some thymocytes. Taken at 3 DPI.

H & E x 250.

Fig. 6: Canine Parvovirus Infection - Lymph node of Dog 5.

In this view of node cortex, there is no distinct follicle and pyknosis and fragmentation of lymphocytes has given a rather "moth eaten" appearance. Intranuclear inclusion bodies were visible at higher powers in this type of field. Taken at 4 DPI.

H & E x 250.

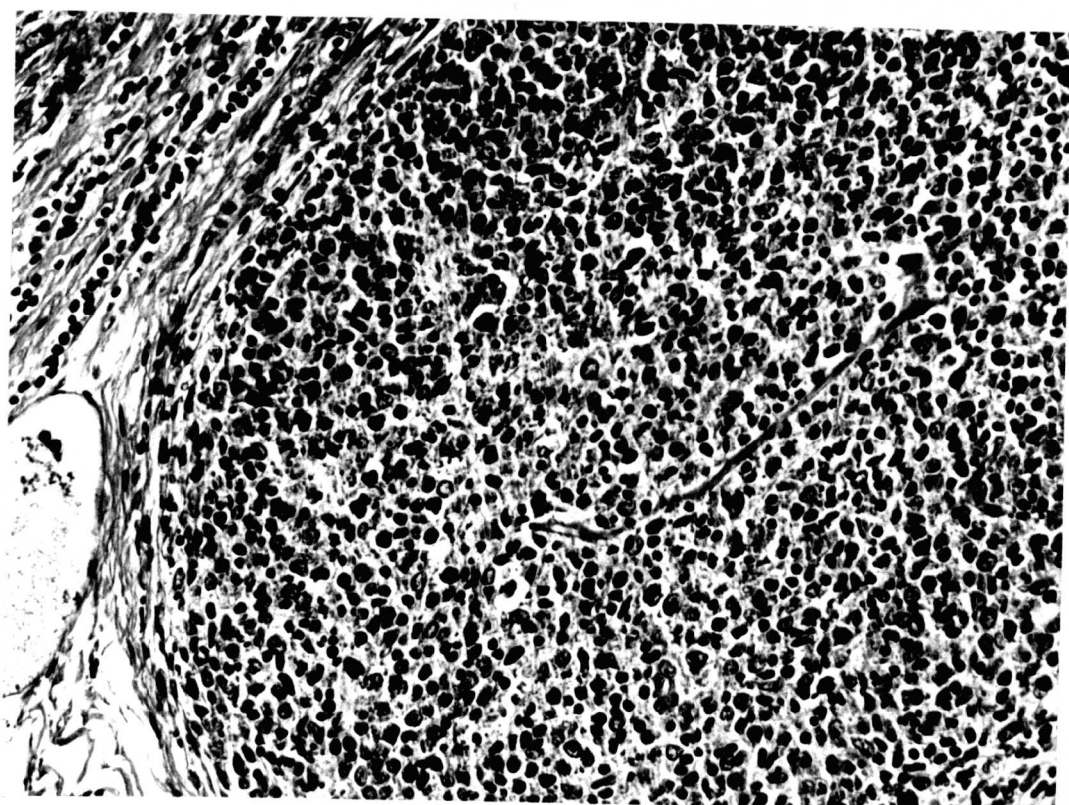
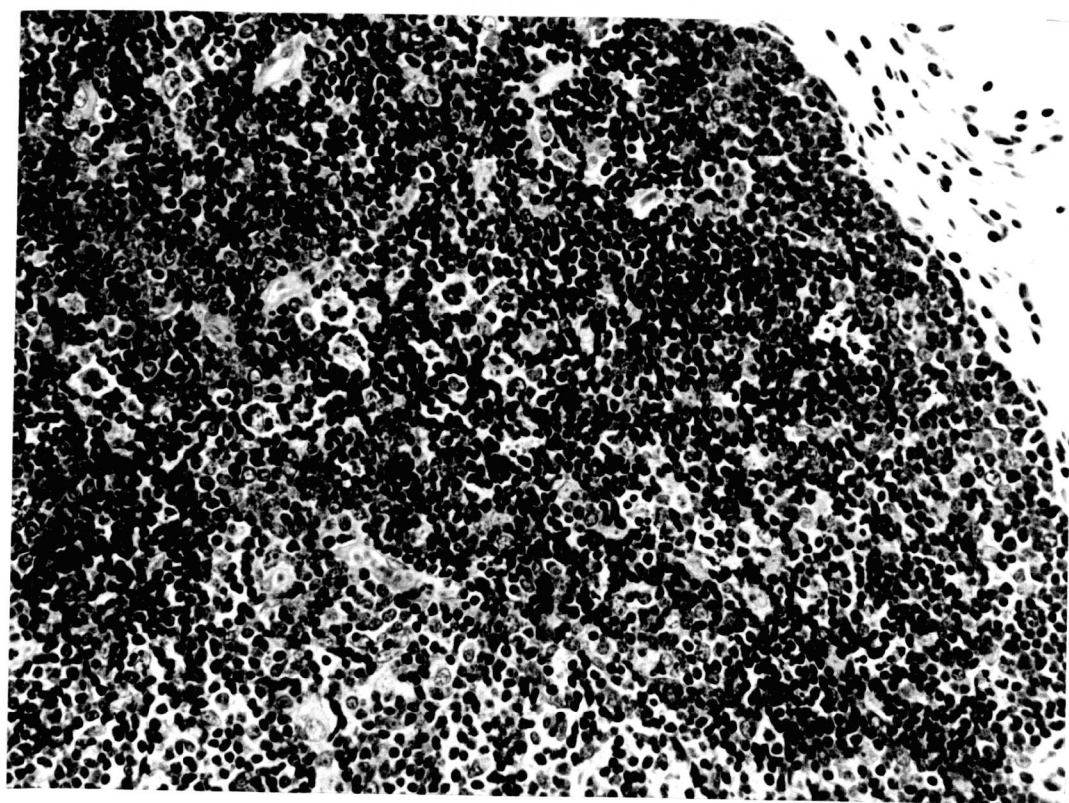


Fig. 7: Canine Parvovirus Infection - Thymus of Dog 6.

The cortex of the thymus is hypocellular, with medulla apparently normal. Taken at 6 DPI.

H & E x 100.

Fig. 8: Canine Parvovirus Infection - Lymph node of Dog 6.

The germinal centre is depleted of centrocytes and is composed mainly of large pale (eosinophilic) cells. Taken at 6 DPI.

H & E x 250.

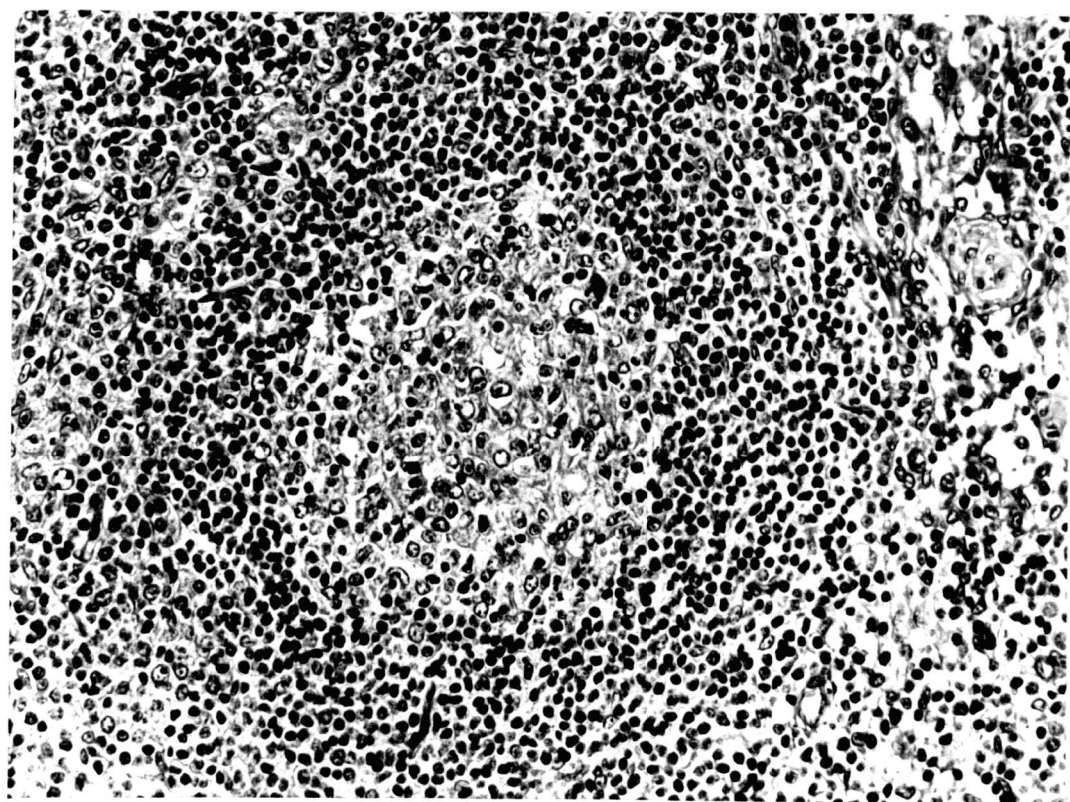
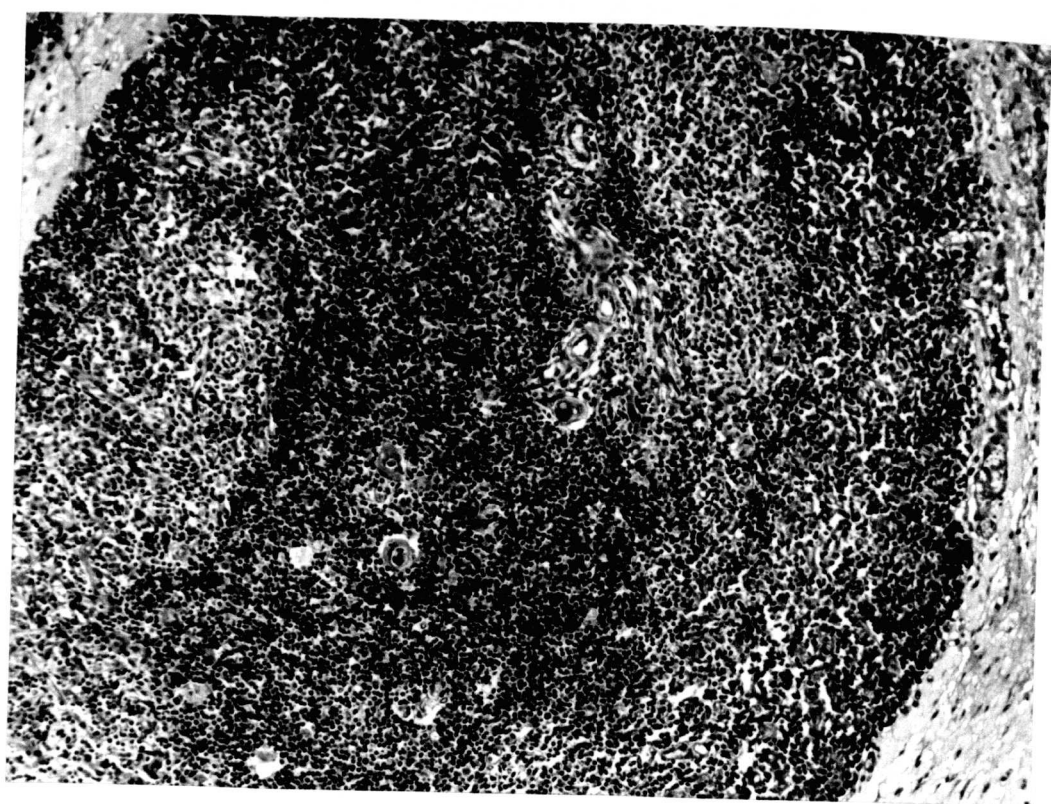


Fig. 9: Canine Parvovirus Infection - Intestine of Dog 6.

The germinal centre of the GALT (arrow) is necrotic being composed largely of amorphous cellular debris. Overlying the GALT, changes are extensive with complete disruption of mucosal architecture. Taken at 6 DPI.

H & E x 100.



Fig. 10: Canine Parvovirus Infection - Intestine of Dog 6.

A non-necrotic germinal centre area of the GALT. An intranuclear inclusion body is arrowed. Taken at 6 DPI.

H & E x 400.

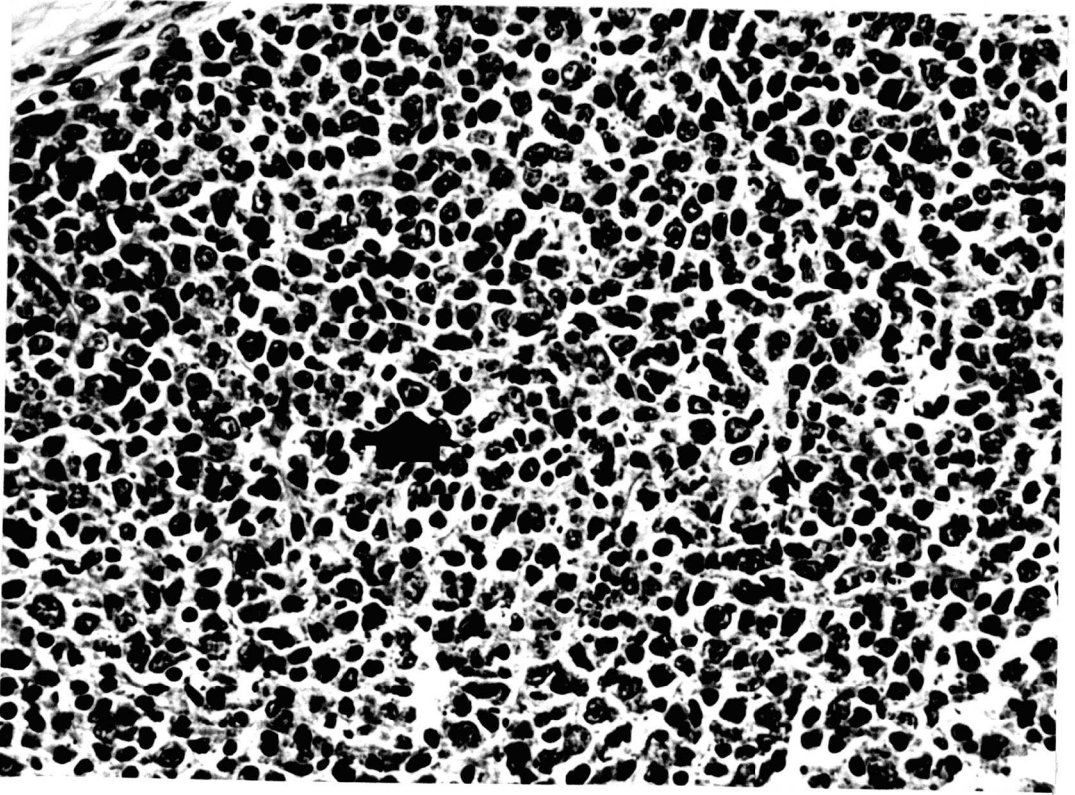


Fig. 11: Canine Parvovirus Infection - Intestine of Dog 6.

There are small, darkly staining cells in the crypt epithelium (small arrow). A more palely staining cell, probably containing an intranuclear inclusion is also present (large arrow). Duodenum, taken at 6 DPI.

H & E x 250.

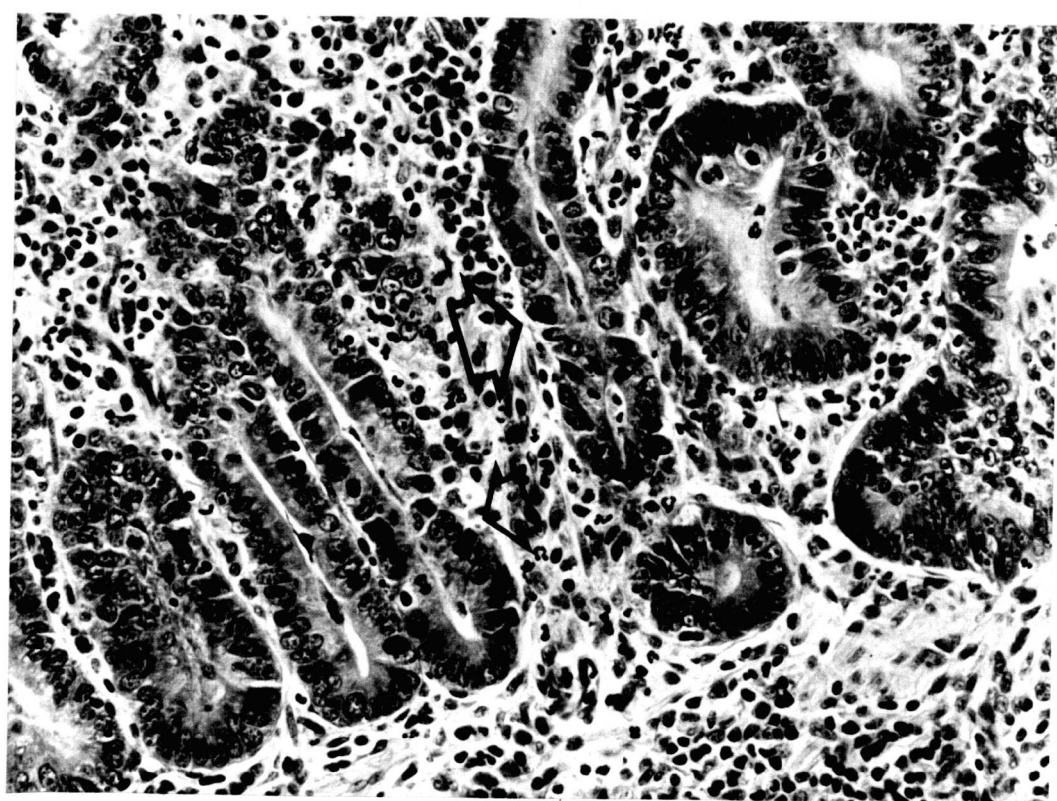


Fig. 12: Canine Parvovirus Infection - Lymph Node of Dog 8.

The germinal centres are repopulated by lymphoid cells. In addition there is expansion of the paracortex with an increased population of plasma cells in the medullary cords visible at higher powers. Taken at 12 DPI.

H & E x 40.

Fig. 13: Canine Parvovirus Infection - Intestine of Dog 9

Note the numerous intranuclear inclusions in crypt enterocytes. The nuclear chromatin appears to be margined to the nuclear membrane, leaving a distinct peri-inclusion halo. Duodenum of contact control dog. Taken at Day 10.

H & E x 400.

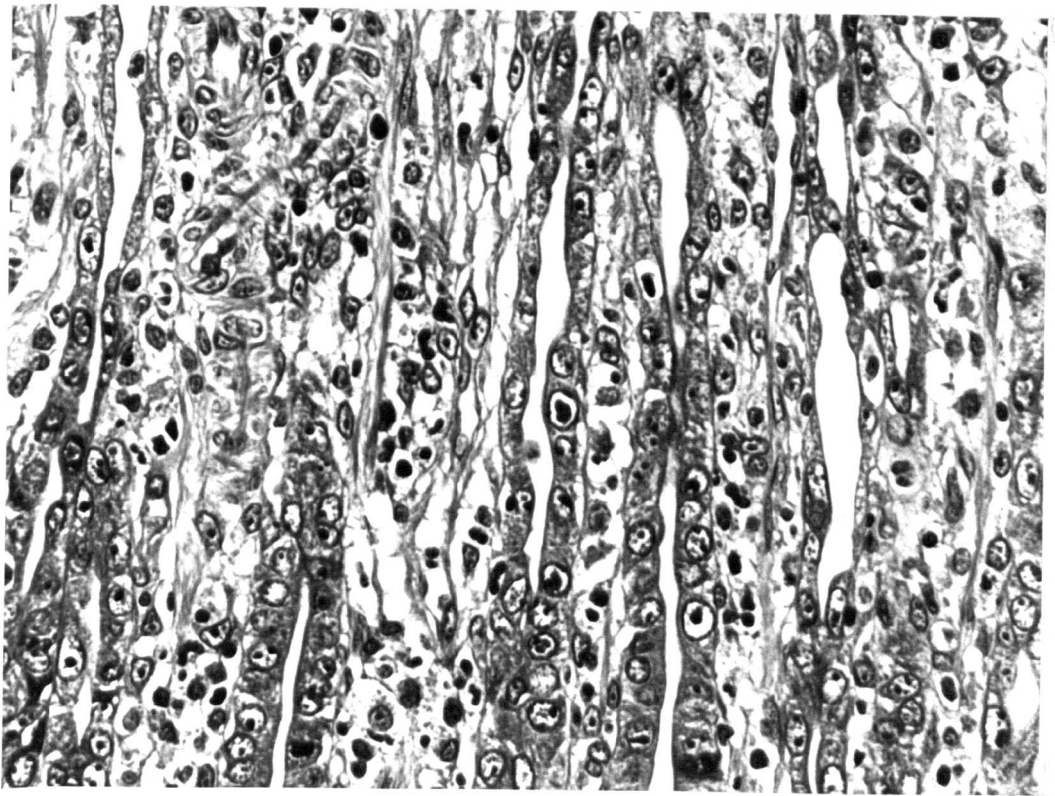
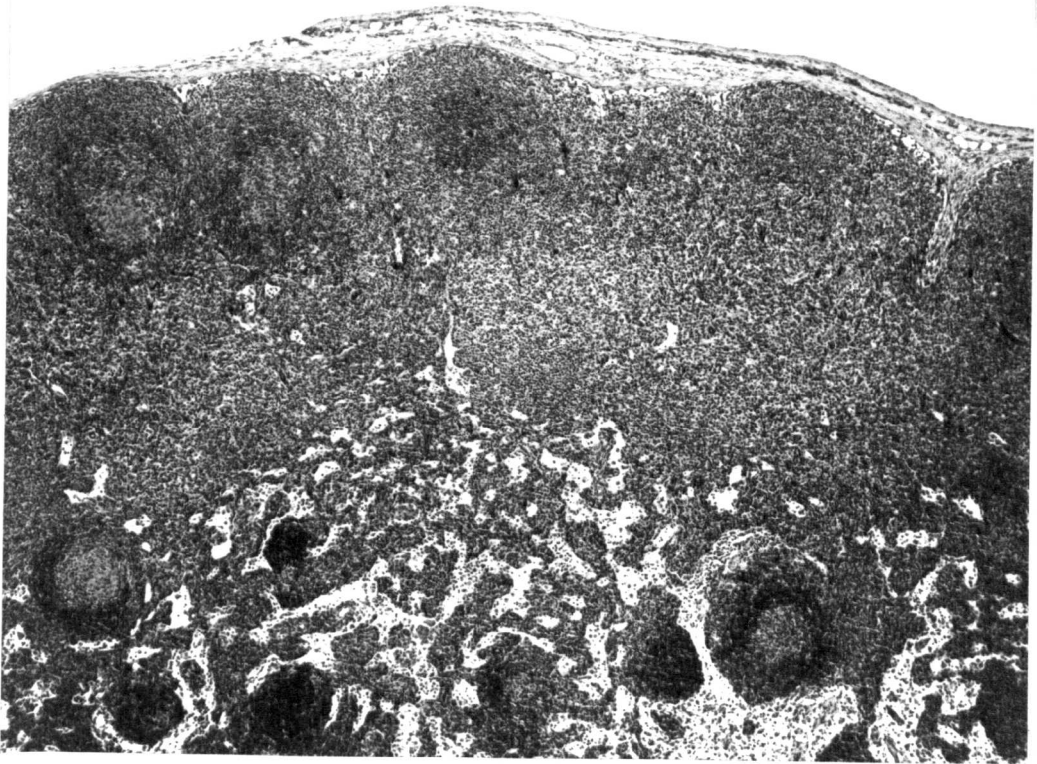
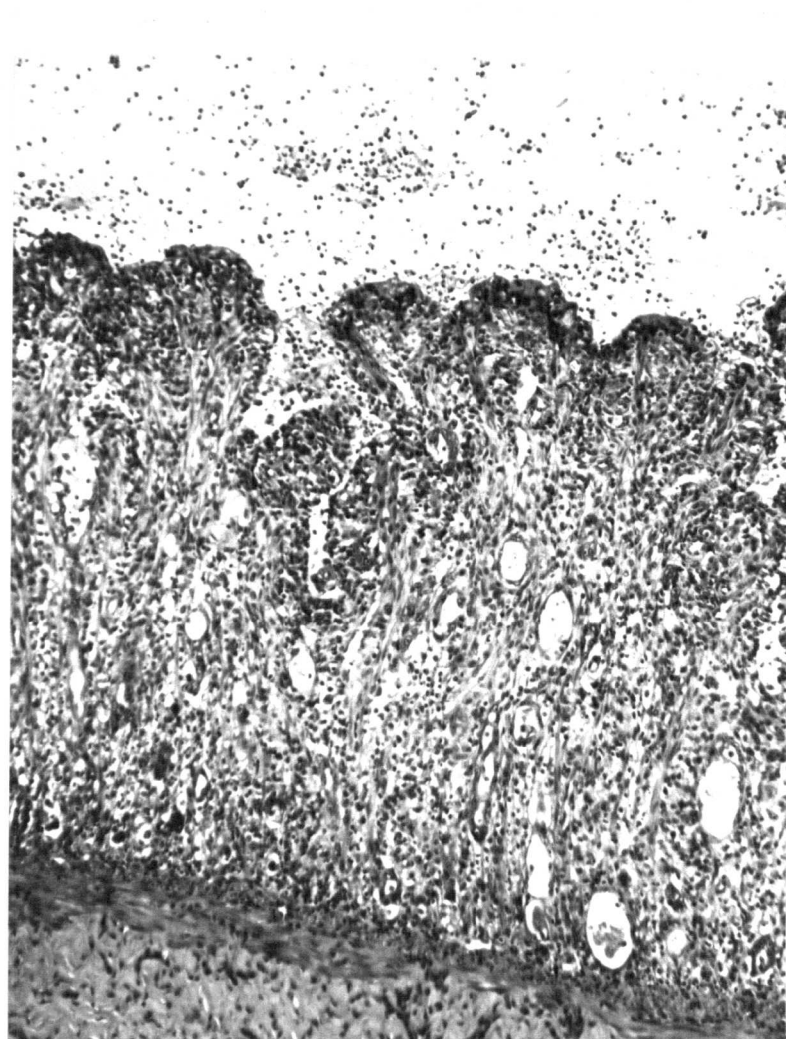


Fig. 14: Canine Parvovirus Infection - Intestine of Dog 9.

There is complete loss of intestinal villi. The crypts are dilated and lined by an attenuated epithelium. The surface epithelium is lost in some places exposing the lamina propria. There is a polymorph infiltrate in the mucosa, with an exudate of neutrophils in the mucus overlying the mucosa. Jejunum, of contact control dog. Taken at Day 10.

H & E x 100.



CHAPTER 4 : PURIFICATION OF CPV FROM FAECES

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INTRODUCTION

In any investigation into infectious disease, the type of challenge material, together with the route of administration of that challenge is important. The obvious route of challenge is that which mimics natural infection and in the experimental reproduction of many canine virus diseases the naturally occurring route of challenge is the one adopted. This is particularly true with non-systemic, locally acting mucosal infections. With canine parainfluenza virus infection, for example, aerosol challenge, which consistently results in respiratory pathology, has been the method of choice for studying the respiratory disease produced by this agent (Appel and Percey 1970). However, in some viral infections, particularly in those which have systemic effects, consistent experimental reproduction of fatal disease is only achieved by use of an artificial route of challenge. For example, to consistently achieve severe clinical disease and death in canine adenovirus Type I infection (infectious canine hepatitis) intravenous challenge must be used since oral challenge with this virus results in a more variable picture (Hamilton et al., 1966).

In canine distemper (CDV), another virus infection with systemic effects, both natural and artificial routes of challenge have been used to reproduce disease. In studies of the efficacy of vaccination against CDV, intracerebral challenge was traditionally used as being a route which consistently resulted in disease and death in non-immune animals (Gillespie and Rickard, 1956) so that the effectiveness of vaccination was readily demonstrable. More recently, the more natural route of challenge by aerosol infection with CDV has been used, particularly in pathogenesis studies (Appel, 1969). In these more recent studies, clinical signs of disease and even death in infected dogs have been reliably reproduced and this may reflect improvements in the techniques of culturing

the virus or of administration of the aerosol challenge compared with those available to earlier workers.

Similarly, with CPV infection, different routes of challenge have been used in the experimental reproduction of the disease. Appel et al. (1979a), infected dogs intravenously with tissue culture derived virus and virus present in filtered faecal material from affected dogs. He noted no clinical enteric disease although lymphopaenia did develop in some animals. Robinson et al. (1980b) also infected dogs intravenously with low passage tissue culture derived virus, but in this study clinical enteric disease with death was observed.

Oral infection with CPV using both virus of tissue culture origin and virus derived directly from affected dogs has also been described. Carmichael et al. (1980) infected dogs orally with $10^{6.2}$ TCID₅₀ of low (2nd) passage tissue culture (Crandell's feline kidney line - CRFK) derived virus but although there was seroconversion, indicating that infection had been successful, clinical signs of illness were not reported. Similarly, McAdaragh et al. (1982) did not observe clinical illness following oral infection with $10^6 - 10^7$ TCID₅₀ of CPV in its 3rd passage in CRFK culture. Azetaka and his coworkers (1981) did record illness in one of three dogs infected orally with CPV in its 3rd passage in CRFK culture while Potgeiter et al. (1981) described severe clinical signs of illness in dogs also orally infected (4×10^4 TCID₅₀ per dog) with CRFK passaged CPV.

Mild clinical disease was also observed following oral infection with CPV obtained from homogenised tissues of affected dogs (Pollock, 1982); but no difference was noted between these dogs and dogs infected with a similar dose ($10^6 - 10^7$ TCID₅₀) of tissue culture derived virus (Pollock, 1982). In contrast, Carman and Povey (1982a and b) observed severe illness in 22 of 24 dogs infected with filtered mucosal scrapings from a naturally occurring case of CPV enteritis.

From these studies it is apparent that the severity of clinical signs following infection with CPV can be highly variable, irrespective of the source of challenge material or the route of administration. Since parenteral challenge with virus of either tissue culture or simple canine derivation does not increase either the constancy or severity of the disease following infection, it would appear to offer no advantage over the more natural, oral route of challenge for investigation of CPV infection and its pathogenesis.

The variability of disease following experimental infection cannot be unexpected in view of the wide range of severity of disease seen in natural outbreaks. Nonetheless, many workers have had great difficulty in reproducing in any way the severe clinical disease pattern which is so typical of the natural infection in pups and a similar situation was encountered in the initial experiment in this study (Chapter 3) where the typical clinical syndrome of naturally occurring CPV infection developed in only one of the dogs (a contact control) in the study.

Many factors are likely to influence the development of severe clinical disease as opposed to asymptomatic infection, but one of the more important of these is likely to be the quality of challenge encountered; a quality which will be determined by the virulence and the amount of virus in the challenge to the animal. Under natural conditions dogs are infected by exposure to the very large amounts of virus present in the faeces of other infected animals. In experimental infections so far, however, dogs have been infected with either tissue culture derived virus at low passage or virus obtained by homogenising and filtering tissues from affected dogs: types of challenge which could differ from "field" challenge either in the nature of virus, the quantity of virus, or both.

The tissue culture derived virus used in the experimental studies described above would not appear to have lost virulence at the low passage levels involved since, in the few studies carried out under directly comparable conditions, no differences were recorded between dogs infected with virus passaged through feline kidney culture and dogs infected with virus derived from tissues of affected dogs (Pollock, 1982). However, the quantity of virus produced in low passage tissue culture systems is limited (Azetaka et al., 1981; Appel et al., 1979a) compared with the amount present in infected faecal material and further passage in tissue culture which could lead to higher virus yields carries the risk of attenuation of the virus. With tissue homogenates, there should be no danger of loss of virulence of the virus, but again the amount of virus present in solid tissues is likely to be lower than that found in faeces of affected animals and there is, moreover, the possibility that adventitious agents might be present.

In view of the resistance of parvoviruses to physical agents, it was considered that a novel approach to the production of high titre, virulent virus might prove feasible, namely purification and concentration by physical methods of such virus from the faeces of naturally occurring cases of CPV enteritis.

Several criteria were set which a suitable method would have to satisfy. Firstly, there should be no significant loss in the amount of infectious virus. Secondly, the virus should be uncontaminated by adventitious agents; and thirdly, the methodology should be simple and economical.

A series of experiments was therefore designed to develop a satisfactory method of purification. Two basic approaches were investigated. In the first, an attempt was made to separate virus from faeces by adsorption onto a solid phase material, hydroxylapatite (HAP). In the second, virus was

separated from other particulate matter in the faeces by density gradient centrifugation.

The HAP method was performed using a variation of the technique described by Smith and Lee (1978) for the large scale isolation of human Type C retrovirus from tissue culture fluid. Virus capsid, which is basically protein, is adsorbed onto HAP in a low ionic strength phosphate buffer at pH 7.4, and subsequently eluted by increasing the ionic strength of the buffer. Adsorption of the viral protein to HAP is largely dependent on interaction between negatively charged moieties on the protein surface and calcium (Ca^{++}) groups on HAP.

Using density gradient centrifugation, two techniques were considered. In the first, the isopyknic technique, the sample is layered onto a preformed density gradient, which encompasses the densities of all particles in the sample. The gradient is formed using Caesium Chloride solutions of increasing concentrations. Under centrifugal force, each particle will sediment to the position in the gradient where the density of the solution is exactly equal to its own. Separation using this technique is on the basis of particle density and is independent of time, although sufficient time must be allowed for each particle to reach its position of isodensity.

In the second method of centrifugation, rate zonal separation, the sample is layered onto a preformed gradient, usually of a viscous solution, such as sucrose. Under centrifugal force, particles will sediment through the gradient at a rate which is largely determined by their mass. To achieve separation using this technique, the densities of the sample particles must be greater than the density of the gradient at any given point, and centrifugation must be terminated before any of the separated zones reaches the bottom of the tube. This method is useful for the separation of particles which have similar densities but which have greatly differing masses.

MATERIALS AND METHODS

Preliminary Preparation of Faeces

Faeces from clinical cases of CPV enteritis were obtained from the samples submitted to the diagnostic service of the Canine Infectious Disease Research Unit at Glasgow University Veterinary School. Samples were selected which contained large amounts of virus (titres in excess of 2048 by the HA test).

Samples for the HAP method were suspended in 0.15 M phosphate buffer whereas those for density centrifugation were suspended in PBS pH 7.2. Heavy particulate matter was removed by ultraclarification, using centrifugation at 20,000 rpm for 30 minutes. For this step, a Sorvall-Du Pont UTD 50 ultra centrifuge was used together with a Sorvall AH 627 rotor (Sorvall-Du Pont, Newton, Connecticut). The optimum time and speed for centrifugation was determined from the results of Experiment 1.

Purification by Hydroxylapatite (HAP)

The hydroxylapatite (Bio-rad Laboratories, Richmond, California) was prepared according to the method of Tiselius et al. (1956). Two grammes of HAP were washed with 80 ml of 0.15 M phosphate buffer, the solid phase allowed to settle, and the supernatant discarded. This washing step was repeated for a second time.

The faecal suspension was added to the HAP slurry and stirred for 18 hours at 4°C. After this adsorption period, the HAP was sedimented by centrifugation at 500 rpm for 5 minutes, and the sedimented HAP washed with 10 ml of 0.15 M phosphate buffer and resedimented by centrifugation. To elute the virus, the sedimented HAP was then suspended in 10 ml of 0.5 M phosphate buffer. The suspension was stirred at room temperature for 30 minutes and the HAP resedimented by

centrifugation. The supernatant fluid was retained and the procedure repeated a further three times; in each, the supernatant was retained.

Phosphate buffers were prepared as follows:

0.15 M Phosphate Buffer, pH 7.4

Stock Solution A	0.15 M Sodium Phosphate, dibasic. (0.465g Na_2HPO_4 made up to 1 litre with distilled water)
Stock Solution B	0.15 M Potassium Dihydrogen Orthophosphate. (9.07g KH_2PO_4 in 1 litre distilled water).

To make up the buffer, 800 ml of Solution A was added to 200 ml of Solution B.

0.5 M Phosphate Buffer pH 7.4

Stock Solution A	1M Potassium Dihydrogen Orthophosphate. (13.6g KH_2PO_4 in 100 ml distilled water).
Stock Solution B	1M Potassium Hydrogen Orthophosphate. (17.4g K_2HPO_4 in 100 ml distilled water).

To prepare the buffer, 19 ml of Solution A was added to 1 ml of Solution B, the pH adjusted to 7.4 with either stock, and the buffer diluted 1:2 to reach 0.5 M ionic strength.

Purification by Density Gradient Centrifugation

All gradients were performed in Beckman Ultraclear tubes (ref. no. 344059, Beckman, Palo Alto, USA) and were then centrifuged in a Sorvall UTD 50 ultracentrifuge using a Beckman SW41 rotor (Beckman, Buckinghamshire). The same type of tube and equipment was used for pelleting the virus.

For isopyknic centrifugation gradients were preformed with solutions of Caesium Chloride of density ranging from 1.2 g/ml (D) up to 1.5 g/ml (D). The solutions were added to the centrifuge tubes by means of 2 mm tubing attached to a syringe. The lightest solution was added first and this was gradually displaced upwards as the heavier solutions were added to the bottom of the tube. The gradients were permitted to diffuse for 24 hours at 4°C before use. Immediately before the addition of the faecal suspension, a protective layer consisting of 1 ml of a 1.20 sucrose solution was gently added onto the top of the caesium gradient in order to prevent disruption of the gradient by the addition of the clarified faecal suspension. A total volume of 5 ml of faecal suspension was then added to each gradient.

For rate zonal centrifugation, a 66% sucrose solution was used. A 'pad' of 1 ml of 1.5D Caesium Chloride was placed at the bottom of the tube, onto which 6 ml of 66% sucrose solution was added, followed by 5 ml of faecal suspension.

After centrifugation, fractions of the gradients were collected by piercing the bottom of the centrifuge tube with a 20 gauge hypodermic needle and collecting the fluid in fractions of ten drops each.

Caesium Chloride (CsCl) solutions were prepared by dissolving the following weights of CsCl salt in 10 ml distilled water.

1.2D	2.77g
1.3D	4.02g
1.4Dg	5.48g
1.5D	6.73g

Sucrose solutions for both rate zonal and isopyknic techniques were prepared by dissolving the following weights of sucrose in 10 ml distilled water.

1.2D	5.26g
1.24D (66%)	6.6g

Electron Microscopical Procedures

Samples of purified virus were prepared for examination in the electron microscope. A drop of virus suspension was applied to a coated grid and left at room temperature for two minutes after which the grid was dried by blotting gently.

One drop of an aqueous suspension of 2% phosphotungstic acid was immediately applied to the grid and left for a further two minutes at room temperature before being blotted dry. The grid was thoroughly air dried, and examined using an AE1 6B electron microscope.

Athene 482 copper grids (Agar Aids, Stanstead) coated with a film of parlodion were prepared as follows:

0.2 ml of 3% parlodion in amyl acetate was added to 0.8 ml amyl acetate. A small drop of the resulting solution was smeared onto a clean glass slide, and the resultant film used to coat the grids.

Haemagglutination Test (HA)

The HA test was performed according to the method described in Chapter 2. The ultraclarified and post-gradient faecal material was not heat treated before being subjected to the test.

Infectivity Titration

FEA cells were seeded into roller tubes in growth medium, each tube being inoculated with 1×10^5 cells. The tubes were stoppered and incubated at 37°C for two hours, when the medium was discarded. 0.2 ml of the test virus suspension, in serial ten-fold dilutions in growth medium was inoculated into the cultures, four tubes per dilution. The tubes were stoppered and incubated for a further two hours at 37°C, after which a total volume of 1 ml of growth medium was added to each tube, and the culture incubated at 37°C for four days.

On the fourth day, each tube was frozen and thawed three times and the supernatant checked for haemagglutinating activity using the HA test. The infectivity titre was taken as the reciprocal of the highest dilution of virus suspensions which permitted detectable virus multiplication in 50% of inoculated cultures.

The growth medium consisted at all stages of Eagle's medium supplemented with 10% FBS as described in Chapter 2.

RESULTS

Experiment 1 : The evaluation of the sedimentation time of CPV in Phosphate Buffered Saline (PBS)

It was important to establish the sedimentation time of CPV in PBS at the start of these studies for a number of reasons. Firstly, pelleting of the virus through saline is, in itself, a simple and economical method of concentrating a viral suspension, since the pellet may be resuspended in a fraction of the original volume. Secondly, the sedimentation time in saline may be used as the baseline timescale for density centrifugation through non-viscous gradients such as CsCl. Thirdly, knowledge of the sedimentation time permits the estimation of optimal conditions for the clarification of a virus suspension without losing significant amounts of free virus.

Experimental Design

After suspension in PBS at a 1:10 dilution, the faecal suspension was centrifuged at 3,000 rpm for 15 minutes to eliminate crude particulate matter. The resultant supernatant was then removed and subjected to the HA test for viral haemagglutinin after which it was divided into three equal aliquots of 12.5 ml. One aliquot was centrifuged at 40,000 rpm for 90 minutes, one was centrifuged at 40,000 rpm for 15 hours, and the third was centrifuged at 40,000 rpm for 18 hours.

Following centrifugation, the supernatant from each tube was decanted, and the pellet suspended in 1 ml PBS by ultrasonication for 15 minutes in a water bath (15°C) followed by vigorous agitation. Both the supernatant fluid and the resuspended pellet were assayed for haemagglutinin and examined by electron microscopy.

Results

The results are shown in Figure 15.

After 90 minutes' centrifugation, there was a large amount of viral haemagglutinin remaining in the supernatant. After 15 hours, there was considerably less in the fluid although low levels were still present. It was not until the tube had been centrifuged for 18 hours that there was complete pelleting of the virus. On electron microscopy parvovirus particles were seen in the pellets but there was also heavy contamination by poorly defined particulate material.

Conclusions

From these findings, it was concluded that for pelleting of the virus in saline, centrifugation at 40,000 rpm for 18 hours would be required. These conditions were subsequently adopted as those necessary for the complete sedimentation of virus in a non-viscous solution.

Experiment 2 : Adsorbtion of CPV onto Hydroxylapatite (HAP)

The aim of this experiment was to determine whether CPV could be separated from faeces by adsorbtion onto hydroxylapatite (HAP).

Experimental Design

The faecal suspension was clarified and then examined for the presence of virus by the HA test and by negative stain electron microscopy. The suspension was adsorbed with HAP and the remaining supernatant checked to ascertain how much virus remained. Virus was then eluted from the HAP by four successive washes and at each stage the quantity and quality of the virus released was checked by haemagglutination and electron microscopy.

Results

The results are shown in Figure 16.

Virus was adsorbed onto the HAP although considerable virus was left in the faecal supernatant. Virus could be eluted from HAP by the 0.5M buffer, with most virus being eluted in the first two washes. On electron microscopical examination, both the first and second washes were contaminated by long filamentous structures approximately 20 nm in diameter and from 100-200 nm in length (Fig. 17). Numerous parvovirus virions were seen. Some of these particles appeared to permit the entry of negative stain into the virion giving the appearance of an 'empty' particle, (i.e. a particle lacking its DNA core) but the majority did not permit the entry of stain and were termed 'full' particles (Fig. 18).

In an attempt to recover more of the virus which remained in the supernatant after the first HAP adsorption, the supernatant was stirred for a further 18 hours with 2g of washed HAP at 4°C. This fresh HAP slurry was then centrifuged and examined as before, but only three 0.5M washes were carried out (Fig. 19).

Following this second adsorption with HAP, more virus was removed and eluted although a considerable amount was still left behind. On electron microscopical examination of the eluted virus, a large number of apparently damaged particles was present. Both empty and full particle types were found but the normally symmetrical appearance of the virions was disrupted and, in addition, some particles had an irregular outline. Contamination by the filamentous structures was again a feature.

Conclusions

CPV was adsorbed onto the hydroxylapatite and this adsorbed virus could be eluted. The eluted virus contained both empty and full particles but was contaminated by filamentous structures. The contaminants were morphologically identical to bacteriophage tails although no intact phage or associated phage heads were observed.

More virus could be recovered from the faecal suspension by additional treatment with HAP but prolonged exposure to HAP appeared to damage the virus.

Experiment 3 : Separation of CPV from faecal suspensions by density gradient centrifugation

The aim of this experiment was to determine whether CPV, with its high density, could be separated from faecal suspensions by density gradient centrifugation.

Experimental Design

Faecal suspensions were clarified by an initial low and subsequent high speed centrifugation. The virus contained in the clarified suspension was then concentrated by ultracentrifugation and resuspended in a small volume of PBS. The antigen contained within the concentrated suspension was then assayed for haemagglutinating activity and checked by electron microscopy.

A) Isopyknic centrifugation

One preparation with a relatively low titre, HA 65536, was layered onto a preformed caesium chloride gradient (Gradient 1); a second preparation with a much higher titre, HA 524,288, was layered onto a second gradient (Gradient 2). Both gradients were centrifuged at 40,000 rpm for 18 hours, examined by

incident light and harvested in fractions of 10 drops each. Each fraction was examined by HA test and electron microscopy.

B) Rate zone centrifugation

Fractions from Gradient 2, found at electron microscopy to be contaminated, were pooled, diluted 1:5 with PBS and layered onto 66% sucrose gradients (Gradients A and B). The loaded gradients were centrifuged at 40,000 rpm for 90 minutes when 10 drop fractions were again collected and assayed by HA and electron microscopy.

C) Infectivity assay

Fractions collected from isopyknic Gradient 1 and rate zonal Gradient A were titrated to establish their content of infectious CPV as described in the Materials and Methods above.

Results

a) Isopyknic centrifugation

The results are shown in Fig.20. On examination of Gradient 1 using incident light, two distinct light refractive bands were observed (Fig.21). Following fraction collection and examination, high haemagglutinin titres were found in fractions 4, 5 and 6 and in each of these parvovirus particles were identified by electron microscopy. Fraction 4, which corresponded to the lower band seen with incident light, contained mainly full virus particles, whereas fractions 5 and 6 corresponding to the upper light refractive band contained a larger proportion of empty virus particles. In addition, fraction 6 was contaminated by filamentous structures, identical to the bacteriophage tails observed in the previous HAP study. No other type of contaminant or any other viruses were observed.

On incident light examination of Gradient 2, no distinct banding was observed (Fig.22), but the gradient was generally cloudy and irregular filaments were present at the level of the 1.4D solution. Following fraction collection from this gradient, the highest haemagglutinin titres were detected in fractions 3-8 and in each of these fractions contaminating filamentous bacteriophage tails were observed by electron microscopy. The contaminated fractions 3-8 were pooled, diluted in PBS and subjected to rate zonal centrifugation.

b) Rate zonal centrifugation

The results are shown in Fig.23.

A mass of filamentous material was present at the interface between the sucrose solution and the basal caesium chloride pad in both Gradients A and B. Following collection of the fractions, high titres of haemagglutinin were detected in fractions 2-6 of Gradient A and fractions 2-4 of Gradient B. Parvovirus particles were observed in all these fractions by electron microscopy but no contaminating material was found. In neither gradient did there appear to be any difference in the proportion of full and empty virus particles found in any of the fractions.

c) Infectivity assay

The results of the infectivity assays on Gradient 1 and Gradient A are shown in Table 11.

In Gradient 1, the greatest infectivity was found in fraction 4, the fraction containing uncontaminated predominantly full (i.e. complete) virus particles. Lower infectivity titres were found in fractions 5 and 6, fractions with the same HA titres as fraction 4 but which on electron microscopy were found to contain a smaller proportion of full virus particles. It is interesting that fraction 3, despite having a much lower HA

titre than fractions 5 and 6 had a similar infectivity titre, indicating that the virus present in fraction 3 must have been composed almost entirely of full, complete virus particles.

In Gradient A, the infectivity titres of the fractions correlated directly with the HA titres apparently reflecting the failure of this gradient to separate full and empty virus particles.

Conclusions

From the results detailed above, it would appear that isopyknic centrifugation of clarified faecal suspensions can yield purified CPV, as exemplified by Gradient 1. Moreover, this technique appears to separate full and empty (i.e. infectious and non-infectious) virus particles.

Contaminated virus obtained by isopyknic centrifugation can be readily purified by subsequent rate zonal centrifugation, the resultant virus still being infectious.

DISCUSSION

At the beginning of this series of experiments, three criteria were established which any purification technique should fulfill. Briefly, these were: that there should be no significant loss of virus; that any virus produced should be uncontaminated; and that the method should be simple and economical.

Simple sedimentation of CPV through PBS allowed concentration of virus from a faecal suspension but did not remove contaminant material.

Adsorption onto HAP with subsequent elution of adsorbed virus proved disappointing as a method both of concentration and of purification. The HAP, in the amounts used, was unsuccessful in completely removing all CPV particles from the original clarified faecal suspensions, so that there was an effective loss of virus. Increasing the amount of HAP used to increase the amounts of virus absorbed would have rendered this method totally uneconomical and attempts to increase virus adsorption by repeated exposure to HAP resulted in unacceptable damage to the virus particles. Moreover, all the virus suspensions produced by elution from HAP were still heavily contaminated with the filamentous, bacteriophage material present in the original solutions, and it is this inability of the HAP method to yield uncontaminated virus which is its most unacceptable feature.

In contrast, the ultracentrifugation techniques investigated appeared to fulfill all the criteria above. Isopyknic centrifugation on its own was extremely effective in concentrating and purifying virus from faecal suspensions of relatively low titre as shown by Gradient 1. However, the isopyknic technique was not as successful when applied to faecal suspensions of high titre as was apparent from Gradient 2. The failure of Gradient 2 to produce the comparatively tight banding of virus seen with Gradient 1 may well have been due to the very large amount of virus used on this gradient resulting in a simple overload. The heavy contamination present in the faecal suspension on this gradient may also have contributed to the poor banding observed.

The potential problem of overload of isopyknic gradients could be easily avoided by limiting the titre of faecal suspensions used on such gradients to a level comparable to that used on Gradient 1. Any residual contamination of virus suspensions present following isopyknic centrifugation could then be easily removed by subsequent rate zonal centrifugation as with Gradients A and B which yielded pure infectious virus.

The main limitation to both the above density gradient techniques is the high capital cost of an ultracentrifuge. However, given that such a piece of equipment is available, the techniques are both straightforward and economical. Sequential clarification, isopyknic and rate zonal centrifugation of faecal suspensions would therefore appear to be a practical method for obtaining pure infectious virus which can be concentrated to any required level by simple pelleting.

For the production of challenge material to be used in future investigations of the pathogenesis of CPV, the following procedure was therefore adopted.

Faecal material taken from dogs clinically affected with CPV enteritis and shown to have a high viral haemagglutinin titre was diluted 1 in 10 with PBS. The suspensions were clarified at 3,000 rpm for 15 minutes and then at 20,000 rpm for 30 minutes. The resulting supernatant was pelleted at 40,000 rpm for 18 hours at 4°C. The pellets were resuspended in 1 ml of PBS using agitation and ultrasonication for 15 minutes. The pooled resuspended pellets were then subjected to the HA test and diluted such that the final concentration was not more than 65,536. 5 ml of this suspension was layered onto an isopyknic gradient preformed to range from 1.2 - 1.5D, and already diffused at 4°C for 18 hours. The loaded isopyknic gradient was then centrifuged at 40,000 rpm for 18 hours at 30°C. The gradient was collected by piercing the bottom of the tube with a 20 gauge needle and the gradient fractions collected in 10 drop aliquots. All fractions were assayed for haemagglutinating activity and fractions with a titre of 4096 and over examined by negative stain electron microscopy. Pure fractions were stored at -20°C until required.

Contaminated fractions were diluted 1:5 with PBS, loaded onto 66% sucrose gradients and subjected to rate zonal centrifugation at 40,000 rpm for 90 minutes. The sucrose gradient was again harvested in aliquots, checked for

haemagglutinin activity and purity and then pelleted at 40,000 rpm for 18 hours. The virus pellets were resuspended in 1 ml PBS and stored at -20°C.

When required for use in oral challenge experiments, the purified, frozen virus was thawed, assayed for haemagglutinin and infectivity and diluted to the required concentration.

TABLE 11.

PURIFICATION OF CPV FROM FAECES -
INFECTIVITY TITRATION OF CPV FOLLOWING
DENSITY GRADIENT CENTRIFUGATION

Fraction	INFECTIVITY TITRE	
	Gradient 1	Gradient A
1	N.D.	N.D.
2	N.D.	2.5 log ₁₀ TCID ₅₀ /ml.
3	2.5 log ₁₀ TCID ₅₀ /ml.	2.5 log ₁₀ TCID ₅₀ /ml.
4	5.25 log ₁₀ TCID ₅₀ /ml.	4.5 log ₁₀ TCID ₅₀ /ml.
5	2.0 log ₁₀ TCID ₅₀ /ml.	-ve
6	2.0 log ₁₀ TCID ₅₀ /ml.	-ve
7	1.25 log ₁₀ TCID ₅₀ /ml.	-ve
8	-ve	N.D.
9	N.D.	N.D.
10	N.D.	N.D.
11	N.D.	N.D.
12	N.D.	N.D.

N.D. = Not Done

Fig. 15: Purification of CPV from faeces - Sedimentation
time of CPV in PBS at 40,000 rpm.

FAECAL SUSPENSION

↓
CLARIFIED BY
LOW SPEED
CENTRIFUGATION

↓
SUPERNATANT

HA Titre

Pellet Supernatant

0 Hours - > 4096

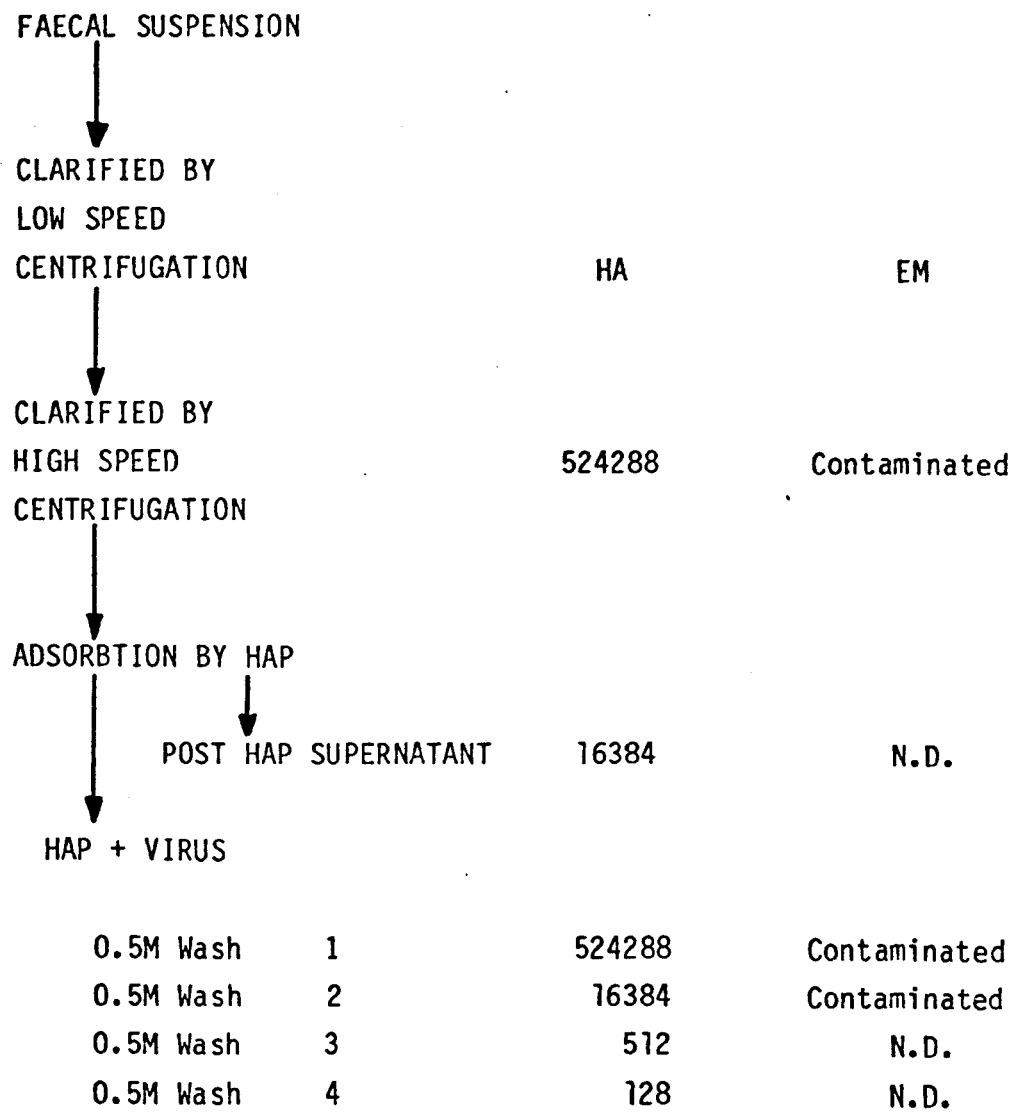
1.5 Hours > 4096 2048

15 Hours > 4096 16

18 Hours > 4096 4

↓
ULTRACENTRIFUGATION

Fig. 16: Purification of CPV from faeces - Adsorbtion
of CPV by Hydroxylapatite (HAP)



N.D. = Not Done.

Fig. 17: Purification of CPV from faeces -
Filamentous Structures

Several filamentous structures approximately 20 nm in diameter may be seen.

x 40,000.

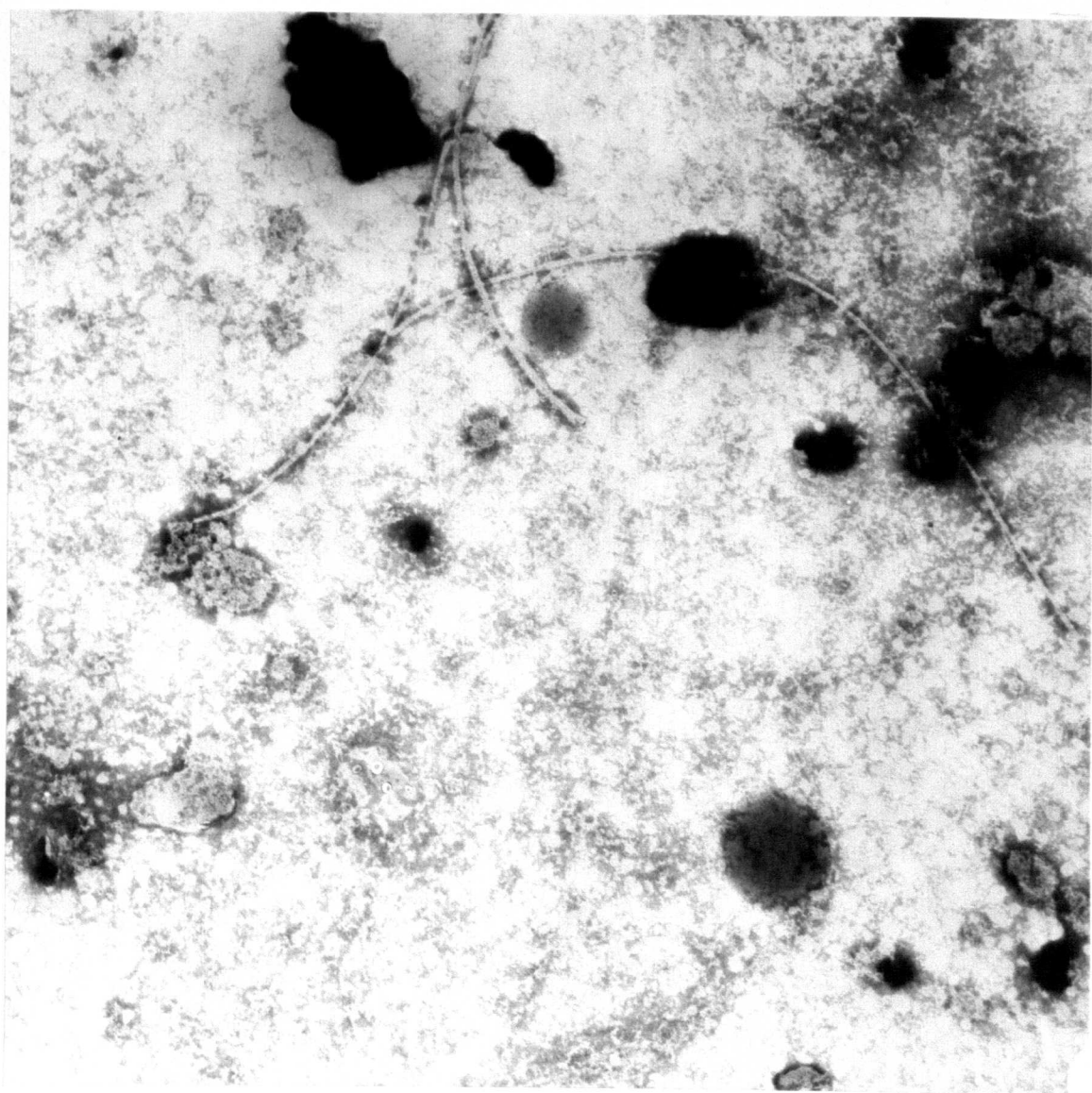


Fig. 18: Purification of CPV from faeces -
Canine Parvovirus.

Many virus particles may be seen. The majority do not permit the entry of stain, while some do, and are termed 'empty particles'.

x 160,000.

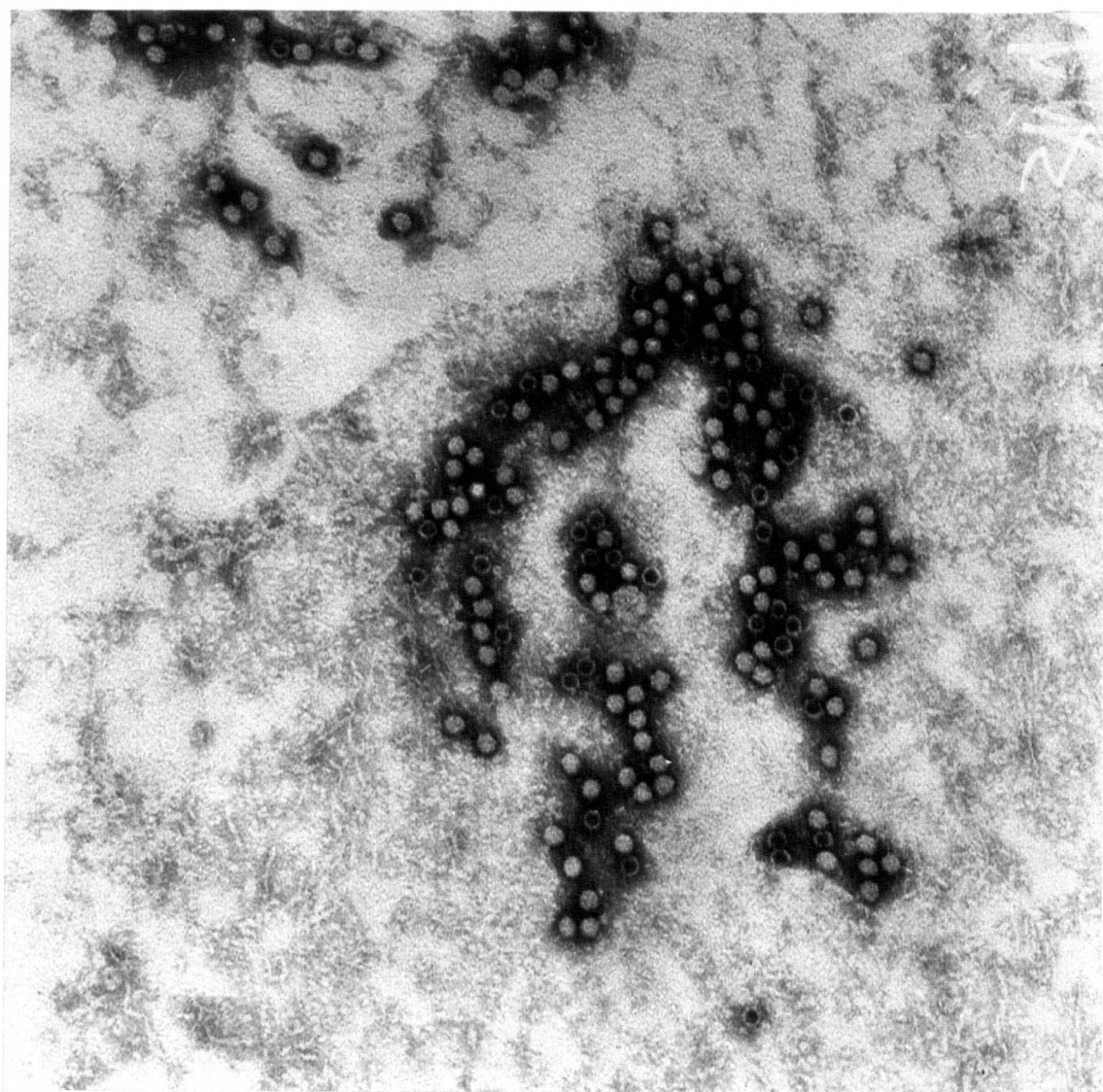


Fig. 19: Purification of CPV from faeces - Repeat
adsorbtion of CPV by Hydroxylapatite (HAP)

POST-HAP
 SUPERNATANT



ADSORPTION BY HAP

HA

EM



SUPERNATANT

2048

N.D.

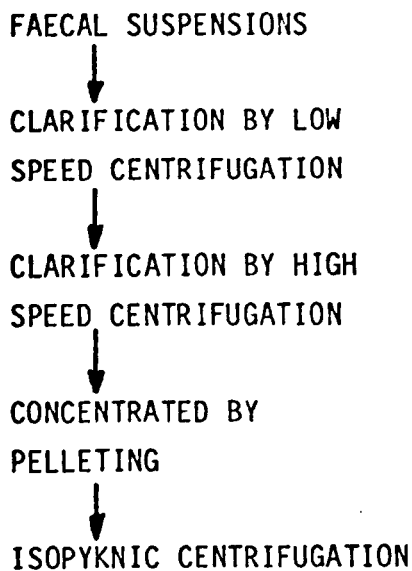


HAP + VIRUS

0.5M Wash	1	8192	Contaminated, Damaged
0.5M Wash	2	2048	Contaminated, Damaged
0.5M Wash	3	128	N.D.

N.D. = Not Done.

Fig. 20: Purification of CPV from faeces - Isopyknic centrifugation of faecal suspensions



Fractions	Gradient 1		Gradient 2	
	HA	EM	HA	EM
1	4	N.D.	4	N.D.
2	4	N.D.	4	N.D.
3	64	N.D.	> 4096	Contaminated 90% complete virus
4	> 4096	Clean, complete virus	> 4096	Contaminated 70% complete virus
5	> 4096	Clean, 70% complete virus	> 4096	Contaminated 50% complete virus
6	> 4096	Contaminated, 70% empty virus	4096	Contaminated 50% complete virus
7	32	N.D.	4096	Contaminated 50% complete virus
8	16	N.D.	4096	Contaminated 70% empty virus
9	8	N.D.	512	N.D.
10	8	N.D.	512	N.D.
11	8	N.D.	256	N.D.
12	8	N.D.	128	N.D.

N.D. = Not Done.

Fig. 21: Purification of CPV from faeces -
Density Gradient Centrifugation, Gradient 1.

Two distinct light refractile bands may be seen (arrows). On electron microscopical examination the lower band is composed predominantly of full particles while the upper consists mainly of 'empty' virions.

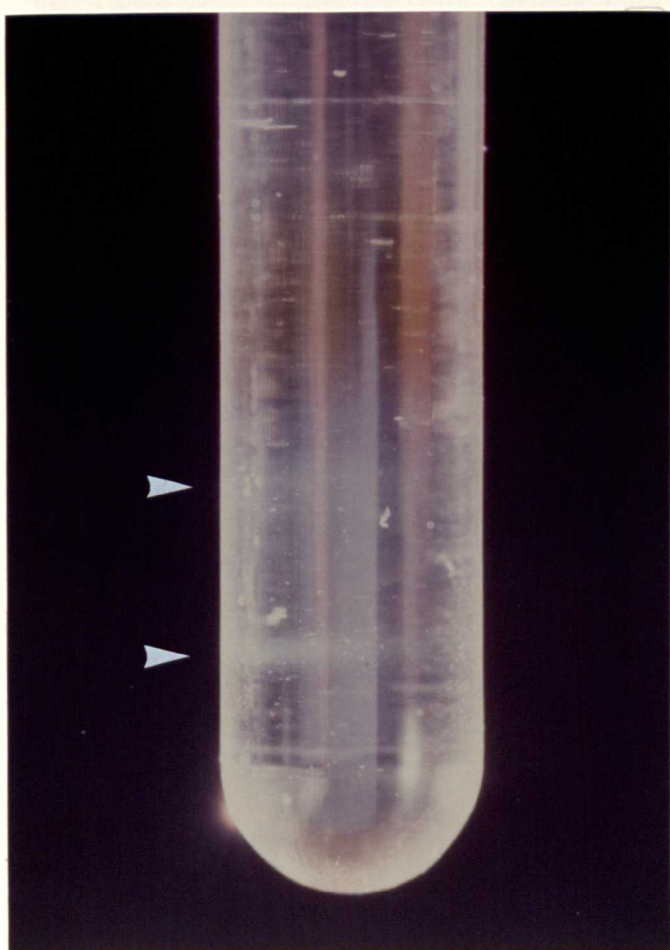


Fig. 22: Purification of CPV from faeces -
Density Gradient Centrifugation, Gradient 2.

The gradient is generally cloudy at the region of 1.40 (arrow). In addition irregular filamentous structures may be seen at this level.

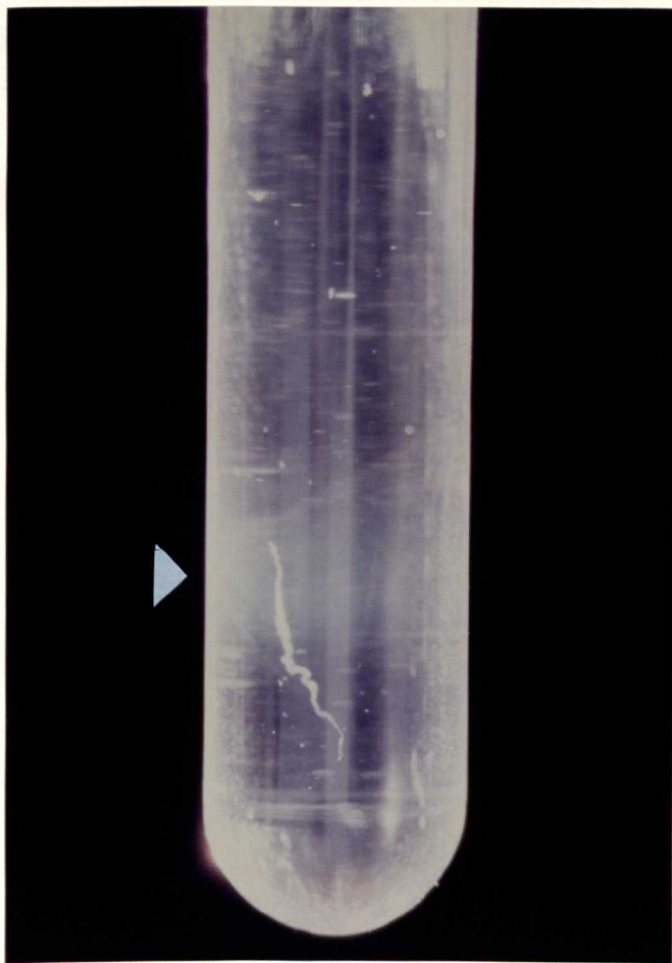
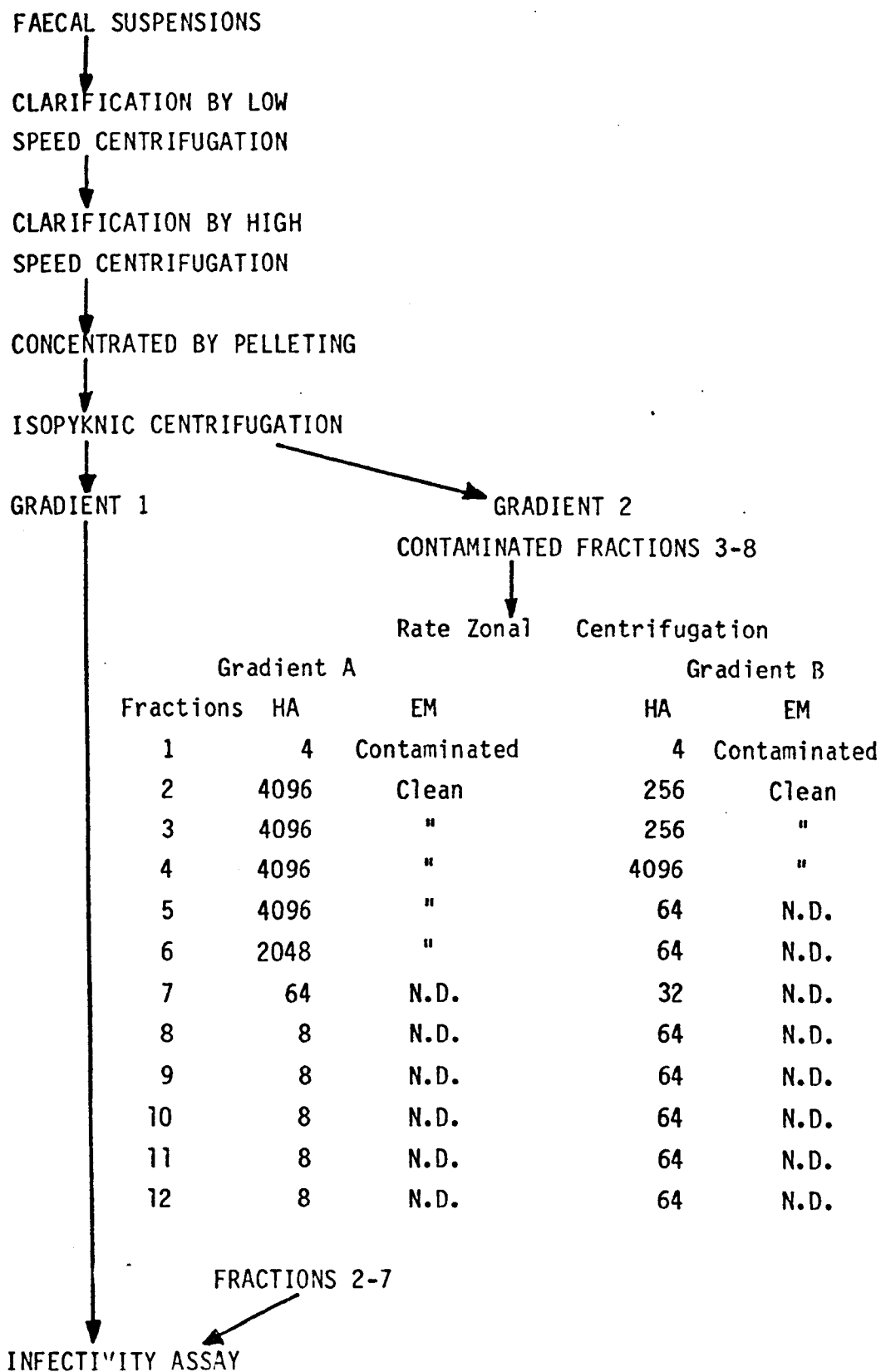


Fig. 23: Purification of CPV from faeces - Rate Zonal
Centrifugation of contaminated CPV Fractions from
Isopyknic Gradients



CHAPTER 5 : DEVELOPMENT OF IMMUNOCYTOCHEMISTRY TECHNIQUES

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INTRODUCTION

From the observations made in Chapter 3 above, it was apparent that conventional histopathological techniques would permit only a limited understanding of the development of lesions following infection with CPV. In order to fully understand the pathogenesis, it would be necessary to develop techniques which would permit the visualisation of viral antigen in tissues so that the distribution of virus during the course of the infection could be studied and related to the development of histopathological changes. Of the numerous immunocytochemical methods available for visualisation of antigens, two techniques, immunofluorescence and immunoperoxidase, were considered and their application to CPV infection investigated.

The pioneering work on immunofluorescence was done by Albert Coons and his coworkers in the early 1940s (Coons et al., 1941), in a study in which they described the visualisation of pneumococcal antigens. It is surprising, in view of the success which this technique was to have in later years, that the methods described were not widely pursued at that time. It was not until the publication of a second paper, some ten years later (Coons and Kaplan, 1950), in which improved techniques for staining and conjugation were described, that the potential for immunofluorescence was recognised. Immunofluorescence has since been widely used in investigations into cell mediated immunity, antibody formation, tissue reactions to foreign organisms and autoimmune disease syndromes (Sternberger, 1974). It has also been applied to the investigation of viral diseases and has been employed in the study of the pathogenesis of almost every major infectious disease of animals. For example, Schipper and Chow (1968) used immunofluorescence to demonstrate Infectious Bovine Rhinotracheitis virus in cases of respiratory disease of cattle when the aetiology was uncertain and Wellemans (1977) used

immunofluorescence to locate Bovine Respiratory Syncytial virus in the lungs of pneumonic calves. Among the numerous enteric viral infections to which the technique has been applied, Pensaert et al. (1970) and Mebus et al. (1973) correlated the distribution of viral antigen to histological lesions in corona virus infections of the pig and calf respectively,

In the dog, Wright and Burns (1966) applied immunofluorescence to the study of Canine Adenovirus infection and described its application as a diagnostic technique. Fluorescent staining was found in the nuclei of the hepatic and Von Kupffer cells containing characteristic intranuclear inclusion bodies. However, specific fluorescence was also observed in acute cases with few or no intranuclear inclusion bodies and this would suggest that the diagnosis might have been overlooked had it been based on conventional histological examination alone.

Immunofluorescence has also proved useful as a diagnostic technique for Canine Distemper Virus infection (Fairchild et al., 1967; Motohashi et al., 1969) using acetone fixed smears of conjunctival, genital or nasal epithelial cells. This type of sample has been shown to be of value in the diagnosis of the infection since it is difficult to transport viable material to laboratories for virus isolation. In his definitive paper on the pathogenesis of Canine Distemper Virus infection, Appel (1970c) applied immunofluorescence and was able to correlate the distribution of viral antigen to the development of lesions in epithelial, lymphoid and nervous tissues. A specific feature demonstrable only by immunofluorescence was the persistence of antigen in the foot pads of dogs in the presence of high levels of circulating antibody.

In the field of canine enteric infections, Keenan et al. (1976) reported the application of immunofluorescence to the study of Canine Corona Virus infection. Fluorescence was most

intense in the epithelial cells at the tips of the villi and there was an accurate correlation between the histological lesions and this distribution of virus antigen.

McAdaragh et al. (1982) have, as yet, been the only workers to apply immunofluorescence to the study of experimental CPV infection. However, only intestinal tissue was examined and no attempt was made to study the sequential localisation of virus during the course of the infection or to relate this to lesions in the lymphoid and alimentary tissues. Immunofluorescence has, however, been applied to the diagnosis of CPV infection in the field. Nelson et al. (1979) described the appearance of viral antigen in crypt enterocytes in the gut and these workers, together with Appel et al. (1979a), have also applied the technique to virus isolation techniques.

There are some inherent difficulties in the technique of immunofluorescence. The requirement for facilities of incident light fluorescent microscopy is perhaps the most obvious problem; but the impermanence of the preparation, necessitating immediate examination, is a more intractable difficulty and photographic facilities must be available if a permanent record of the result is required. Moreover, in order to minimise loss of antigenicity as a result of tissue processing, frozen sections have been widely used leading to problems of poor resolution and definition in the final preparations. Storage of the frozen blocks is also fraught with difficulty and requires constant attention to prevent dehydration of the tissues or thawing of the blocks with subsequent formation of ice-crystals upon refreezing.

These problems have led several workers to attempt immunofluorescence on paraffin embedded material, but poor reproducibility and an apparent increase in background staining have limited the use of paraffin sections. Where paraffin embedded material has been used successfully, either enzymatic pre-treatment of formalin fixed material (Huang, 1975; Witting,

1977), or the use of coagulative, alcoholic fixatives (Sainte-Marie, 1962; Dorsett and Ioachin, 1978) has been required. In the work to be described in this section, conventional immunofluorescent examination using frozen sections was employed. In addition, immunofluorescence was also attempted in paraffin embedded tissues fixed in a variety of alcoholic and acidic fixatives, as well as 10% NBF.

To overcome the technical problems inherent in immunofluorescent techniques, other, non-fluorescent methods of immunocytochemistry have been developed in recent years. These newer techniques include radiolabelling, Protein-A-Gold labelling and enzyme labelling with both alkaline phosphatase and horseradish peroxidase (Bloom and Polak, 1981). By far the most popular methods have been those using horseradish peroxidase (immunoperoxidase).

There are several methods of immunoperoxidase staining, all of which have been reviewed by Heyderman (1979). The basis of all the techniques is the attachment of the enzyme, horseradish peroxidase, to antigen by means of antibody and the subsequent polymerisation of an electron donor over the enzyme site in the presence of hydrogen peroxide. This yields an insoluble brown polymer at the site of the antigen which can be visualised using light or electron microscopy. Briefly, the methods available are categorised as direct, indirect or unlabelled antibody-enzyme techniques. In the direct method, horseradish peroxidase is coupled to specific antibody, and this conjugate is applied directly to the specimen. With the indirect method, a specific antiserum is incubated with the specimen. Subsequently, horseradish peroxidase conjugated antiserum raised against the species globulin of the original specific antiserum is incubated with the specimen and the enzyme reaction completed. This indirect technique permits the use of one conjugated antiserum with a variety of primary antisera raised in the same species and has been fairly widely used (Wicker and Avrameas, 1969; Burns et al., 1974; Carthew, 1978).

However, both the indirect and direct methods require the conjugation of the peroxidase to the antibody which can markedly reduce the binding capacity of the immunoglobulin. To remove the need for conjugation, Sternberger et al. (1970) developed a technique for the solubilisation of an immune complex of peroxidase and rabbit anti-peroxidase (the PAP complex) and its subsequent use in a staining procedure. This procedure uses a primary antiserum raised in rabbits, a bridging anti-rabbit immunoglobulin and the peroxidase-anti-peroxidase (PAP) complex (Fig 28). The basic advantage of this technique is its increased sensitivity, permitting a higher dilution of the primary antiserum and enabling the demonstration of antigen in tissue fixed and processed by a wide variety of methods, since only small numbers of antigenic sites are required. In Sternberger's original communication, the technique was used to visualise spirochaetes in air dried smears, but the technique has since found wide application in biological investigations and has superseded the direct and indirect methods (Sternberger, 1979). An additional advantage of the PAP method is that retrospective study of stored tissue blocks is possible (Halmi and Duello, 1976).

Polak and Bloom (1978) reported the use of the PAP technique in an investigation of neuroregulatory peptides and found it to permit highly accurate localisation of a variety of neuroactive amines. Elias and Miller (1975) applied it to the study of glomerulonephritis, and were able to demonstrate antibody/antigen complexes and complement in the glomeruli of patients with proteinuria. Curran and Gregory (1978) used both immunofluorescence and the PAP technique to demonstrate immunoglobulin in frozen and paraffin embedded sections. These workers reported poor results using immunofluorescence on formalin fixed tissues, whereas in similar sections examined by the PAP method there was both good specific staining and clear definition of antigen.

In human virus infections, immunoperoxidase has found major application in the localisation of Hepatitis B antigens (Clausen and Thom sen, 1978) and has been used to elucidate the pathogenesis and immunopathology of this important infection. Of particular importance has been the role played by the PAP technique in demonstrating the importance of Hepatitis B antigen in cases of chronic active hepatitis (Gudat et al., 1975; Lamothe et al., 1976) and hepatocellular carcinoma (Turbitt et al., 1977).

Other virus infections which have been investigated using immunoperoxidase include Herpes Simplex and Measles (Kumanishi and Hirano, 1978) and infection with cytomegalovirus (Kurstak, 1971), Epstein Barr Virus (Emura et al., 1978) and SV 40 (Tabuchi et al., 1978). Most of these studies, like those on Hepatitis B, were performed retrospectively, since the PAP technique permitted the use of formalin fixed, paraffin embedded material and produced stained sections which could be examined with a high degree of accuracy.

In contrast to the medical sciences, peroxidase techniques have not been widely applied in veterinary research. Carthew (1978) used immunoperoxidase to permit rapid diagnosis of mouse viral hepatitis in fatal cases from natural outbreaks. Techniques have also been developed for the demonstration of bovine papilloma virus antigens in tissues (O'Neil, 1982), which permitted the differentiation of subtypes of Bovine Papilloma Virus. In the dog, Ducatelle et al. (1980) applied the PAP technique to Canine Distemper Virus infection and described its use as an adjunct to light microscopy in the diagnosis of this infection. These workers claimed that some cases with abundant cytoplasmic inclusions were negative for viral antigen using immunoperoxidase, suggesting that false positive diagnoses might have been returned with histological examination alone. An alternative explanation may be that there was a failure of PAP staining in some cases.

While peroxidase techniques, and the PAP technique especially, have many advantages, some technical difficulties may be encountered with the procedures. Tissue sections require, in most cases, pre-treatment with trypsin to unmask cross linked antigenic determinants. In addition, endogenous peroxidase activity, predominantly found in the granulocytes and erythrocytes, must be abolished before any specific staining may be attempted. However, perhaps the most cumbersome aspect of the PAP method is that three control sections are required for each section stained to ensure absolute specificity of the reaction.

The following study deals with the development and application of immunofluorescence and immunoperoxidase (PAP) techniques to CPV infection.

MATERIALS AND METHODS

Control Tissue

Two types of material were available which were known to contain viral antigen: infected cells in tissue culture and tissues from infected dogs.

The use of tissue culture was attempted and discarded because of the difficulties in retaining cells on coverslips during staining procedures. Sections of lymphoid and alimentary tissues from dogs with CPV enteritis were also considered but since the distribution of CPV antigen in these tissues in relation to their architecture and to the duration of infection was unclear (hence the need for the development of immunocytochemical techniques), it was decided to use sections of myocardium from pups with CPV associated myocarditis as positive control tissue. The advantage of this tissue was that it provided a well-localised focus of viral antigen in the distinctive intranuclear viral inclusion bodies which are found in cardiac myocytes in this condition.

The pup from which the control cardiac tissue was taken had died suddenly at 6 weeks of age. At post-mortem examination, the tracheobronchial tree contained a frothy exudate and the lungs were filled with oedema fluid. The heart was removed and blocks of cardiac muscle fixed using 10% NBF, Bouin's fixative, ethanol, methanol and paraformaldehyde/glutaraldehyde. In addition, portions of heart muscle were snap frozen in liquid nitrogen for cryostat sectioning. Light microscopical examination of formalin fixed material revealed large numbers of basophilic intranuclear inclusion bodies (Fig. 24) in cardiac myocytes, together with interstitial oedema. Ultrastructural examination of blocks fixed in paraformaldehyde/glutaraldehyde revealed that the inclusions were composed of aggregates of round particles 20 nm in diameter, admixed with small perichromatin granules (Fig. 25); the round structures were morphologically identical to CPV virions. This tissue was therefore considered suitable for use as a known 'positive' since the antigen was well localised in structures which could be identified using routine light microscopy.

Histological Procedures

A. Fixation.

10% Neutral Buffered Formalin

All tissue blocks were fixed, trimmed and post-fixed in corrosive formol as described in Chapter 2.

Bouin's Fixative

Tissues were fixed and trimmed using this fixative as described in Chapter 2.

Iced Ethanol

Blocks not more than 5 mm thick were dropped into 95% ethanol at 4°C and refrigerated for one hour after which they were trimmed to between 1-2 mm thick and refrigerated for a further 24 hours. They were then processed as described below.

Methanol

Blocks of tissue less than 5 mm thick were placed in absolute methanol for 24 hours, trimmed to a thickness of 2 mm, and immersed in absolute methanol for a further 24 hours. They were then processed as described below.

Paraformaldehyde/glutaraldehyde

Tissues were fixed as described in Chapter 2.

Snap Frozen Blocks

Unfixed tissue blocks were snap frozen in liquid nitrogen as described in Chapter 2.

B. Processing.

10% Neutral Buffered Formalin, Bouin's Fixative

These tissues were processed as described in Chapter 2.

Iced Ethanol

Trimmed tissue blocks were dehydrated through a series of absolute alcohols, cleared in three consecutive xylene baths at 4°C, and allowed to reach room temperature. They were impregnated in a series of wax baths at 56°C, embedded in fresh

paraffin wax and stored at 4°C until required.

Methanol

Trimmed tissues were cleared in three one hour changes of xylene, and impregnated in three changes of paraffin wax at 56°C, the tissues being permitted to equilibrate to room temperature after each change. Following the third change of paraffin wax, tissues were dried in a vacuum oven for 30 minutes, embedded in fresh wax and sectioned. The cut sections were allowed to stand at room temperature for 18 hours and were then passed through three five minute changes of xylene, followed by a solution of absolute alcohol for a further five minutes. The sections were air dried and allowed to stand at 4°C for a minimum of three hours before staining.

Paraformaldehyde/glutaraldehyde

Blocks fixed in this solution were processed as described in Chapter 2.

Production of Antisera

Two types of primary antisera were used, dog anti-CPV and rabbit anti-CPV.

A. Dog Anti-CPV.

This serum was removed from a dog which had recovered from natural CPV infection. The animal was four months old when the serum was collected. No antibodies to Canine Distemper Virus nor to Canine Adenovirus were detected using neutralisation tests.

B. Rabbit Anti-CPV

Six young healthy rabbits were selected. A minimum of 60 ml of blood was removed from each rabbit and the separated serum collected. This pre-immunisation serum was subsequently used as negative control serum for each specific rabbit serum produced.

The rabbits were then primarily immunised by injecting 1 ml of an emulsion of 50% Freund's complete Adjuvant (Miles Labs. Indiana, USA) and 50% of a CPV virus suspension with a minimum HA titre of $1 \times 10^6/0.025$ ml deep into the quadriceps muscle of each hind limb. The virus had been produced as previously described. After six weeks, each rabbit was given an intravenous dose of virus, consisting of 1 ml of purified virus with an HA titre of no more than $1 \times 10^4/0.025$ ml. Six days after the intravenous dose, the animals were anaesthetised by intravenous administration of pentobarbitone sodium (Sagital, May and Baker, Dagenham) and were exsanguinated by cardiac puncture. Blood was allowed to clot and the serum removed and aliquoted before being stored at -20°C until required. The HAI titres of the sera produced are shown in Table 12. The serum of rabbit 3, with the highest HAI titre (1048576), was used in the initial developmental work on immunocytochemical methods.

Conjugation of antisera

For developmental work only dog anti-CPV antiserum was conjugated with fluorescein isothiocyanate (FITC). However, in latter studies, a rabbit anti-CPV antiserum was also conjugated to FITC. Irrespective of the type of antiserum, the following conjugation method was used.

Immunoglobulin was precipitated from the serum by the dropwise addition of an equal volume of saturated ammonium sulphate solution in an ice bath. After mixing in the ice bath

for one hour the precipitate was washed several times with 50% ammonium sulphate to eliminate any haemoglobin. The final washed pellet was resuspended in PBS at 1/3 of the original volume of serum. The resulting solution was then dialysed extensively against PBS to eliminate ammonium sulphate, and the protein concentration of the solution estimated using an ultraviolet spectrophotometer. The absorbance of the solution was read at a wavelength of 260u, and the protein concentration calculated according to the formula.

$$\text{mg. Protein/ml} = \frac{\text{O.D.} \times 100}{1.32}$$

(O.D. = Absorbance)

100 mg. of protein was diluted using a 0.15M solution of sodium chloride, to which was added 1.5 mg of FITC (Sigma, London) dissolved in Carbonate/Bicarbonate buffer pH 9.0. The solutions were thoroughly mixed, the pH checked and brought back to pH 9.0 by the addition, if necessary, of further Carbonate/Bi carbonate buffer. The mixture was stirred at 4°C for 18 hours and unreacted fluorochrome then removed by passing the mixture through a Sephadex G25 fine grade column (Pharmacia, Upsala, Sweden), packed using PBS pH 7.4. The conjugated fraction obtained from the column was absorbed three times with canine liver powder to remove any negatively charged overlabelled macromolecules. The conjugate was then aliquoted and stored at -20°C until required.

Saturated Ammonium Sulphate

This was prepared by dissolving 69.7g of ammonium sulphate salt in 100 ml of ice cold distilled water.

Bicarbonate Buffer pH 9.0 was prepared as follows:

Sodium Bicarbonate	2.93g
Sodium Carbonate	1.59g
Sodium Azide	0.2g
Distilled water	1000 ml.

Tissue Powder

This was prepared by dehydrating minced and homogenised canine liver tissue with repeated large volumes of acetone. The resulting sediment was dried overnight at 37°C before being ground in a pestle and mortar. The powder was stored at -20°C until required.

Immunofluorescent Procedures

Both Direct and Indirect Immunofluorescence was attempted.

Direct Fluorescence

Both frozen and paraffin embedded tissues were used. Frozen sections were prefixed in acetone for 5 seconds. Sections were incubated with the conjugate prepared as described above for 30 minutes at room temperature. Sections were then washed in three changes of PBS of 15 minutes each with constant agitation, after which they were mounted using a solution of 50% glycerol in PBS.

Indirect Fluorescence

Both frozen and paraffin embedded tissues were used and two sections were cut from each tissue block to be examined. Frozen sections were fixed in acetone for 5 seconds. One of the sections was incubated with the specific rabbit anti-CPV antiserum (undiluted) and the other with the pre-immunisation serum from the same animal. Both sections were incubated for

30 minutes at room temperature after which they were washed in three changes of PBS of 15 minutes each, under constant agitation. Following washing, all sections were incubated with FITC conjugated goat anti-rabbit IgG (Sigma, London) at the dilution recommended by the manufacturer for 30 minutes at room temperature. Sections were then washed and mounted as for direct immunofluorescence.

Immunoperoxidase Procedures

For developmental work, the method described by Sternberger (1974) was initially used and is described below.

Immunoperoxidase Method (PAP (Sternberger, 1974)

Formalin fixed, paraffin embedded and frozen sections were used.

1. Paraffin embedded sections were hydrated using a series of solutions of xylene, absolute alcohol, methylated spiritis, 70% ethanol and finally water. The sections were then immersed in Lugol's iodine for 5 minutes followed by immersion in 5% sodium thiosulphate solution to remove mercuric pigment. After this, sections were washed well in running tap-water.
2. Both paraffin sections and freshly cut frozen sections were then washed in two changes of absolute alcohol, of 5 minutes each.
3. Sections were incubated with 0.5% hydrogen peroxide in methanol at room temperature for 30 minutes.
4. Sections were washed in Tris buffer, pH 7.6 for 5-10 minutes followed by a second rinse in Tris buffer with 1% normal sheep serum for 10 minutes.

5. Sections were incubated with rabbit anti-CPV antiserum diluted in PBS to the optimal dilution determined for immunofluorescence, for 30 minutes at room temperature.

6. Step four was repeated.

7. Sections were incubated with 1:20 dilution of sheep anti-rabbit IgG antiserum (Sigma, London), diluted in PBS, for one hour at room temperature.

8. Step four was repeated.

9. Sections were incubated with a 1:20 dilution of peroxidase/anti-peroxidase raised in rabbit (Mercia-Brocades, Weybridge) for one hour at room temperature.

10. Step four was repeated.

11. Sections were immersed in a solution of 25 mg diaminobenzidine (DAB) (Sigma, London) dissolved in 50 ml Tris/HCl buffer, with 3% hydrogen peroxide. They were incubated in this solution for 5 minutes at room temperature.

12. Sections were washed in running water, and routinely counterstained with haematoxylin.

13. The sections were dehydrated and mounted routinely (Chapter 2).

For each section subjected to the entire staining procedure described above, the following control sections were also examined.

Control One. This section was subjected to the above staining procedure. The pre-immunisation serum from the rabbit was substituted for the specific anti-CPV antiserum.

Control Two. This section was subjected to the above staining procedure, except that it was not incubated with the anti-CPV antiserum nor with the bridging sheep anti-rabbit antibody. This ensured that there was no cross-reactivity between the rabbit peroxidase anti-peroxidase and antigens on the tissue section.

Control Three. This section was subjected to the above staining procedure except that it was not incubated with the anti-CPV antiserum, the bridging sheep anti-rabbit antibody nor the PAP complex. This control section was designed to ensure that elimination of endogenous peroxidase was complete.

In all developmental work formalin fixed corrosive post-fixed blocks of the control tissue were used, with the exception of Experiment 2 where fresh frozen sections were used.

Tris Buffer pH 7.6

Stock solutions of this buffer were prepared as follows:

Sodium Chloride	116.9g
Trisma Base	24.2g
Potassium EDTA	6.7g
Distilled water	1900 ml.

The pH was adjusted to 7.6 with concentrated HCl, and the total volume made up to 2000 ml. For use, this stock solution was diluted 1:10 with distilled water.

RESULTS

Experiment 1 : Development of immunofluorescence techniques

Both direct and indirect methods of immunofluorescence were examined.

Experimental Design

Sections prepared from blocks fixed in 10% NBF (postfixed in corrosive formol), Bouin's fixative, Iced Ethanol and Methanol along with frozen sections were examined by the direct and indirect immunofluorescence methods described.

Subsequently, using the indirect technique, serial frozen sections of control cardiac tissue were incubated with serial 1 in 10 dilutions of the primary, unconjugated rabbit anti-CPV antiserum in PBS (from undilute to 1 in 10^4). All six rabbit anti-CPV antisera were tested in this way. The commercial FITC conjugated goat anti-rabbit antiserum was used only at the recommended dilution of 1 in 32.

Results

The results are shown in Table 13. Using both direct and indirect techniques, nuclear fluorescence was observed only in sections from the frozen tissue blocks (Fig. 26); there was no cytoplasmic or interstitial fluorescence in these frozen sections and the control sections were negative.

There was no nuclear fluorescence in any section from paraffin embedded blocks. However, in the paraffin sections of the Bouin's fixed tissue, granular cytoplasmic fluorescence was observed in large macrophage-like cells in the interstitium. This cytoplasmic fluorescence was also observed in the control sections (incubated with pre-immunisation sera) of the indirect technique which indicated that it was a non-specific reaction.

The results of the titration to determine the optimal dilution of the primary rabbit anti-CPV-antisera are shown in Table 14. The optimal dilution was that yielding maximal intensity fluorescence of antigen with minimal background staining. In general, the higher the initial HAI titre of the serum, the higher was the optimal dilution.

Conclusions

Focal nuclear fluorescence was observed only in sections from frozen tissue blocks, indicating that the processing involved in fixing and embedding the other blocks had destroyed or masked sufficient antigenic determinants to render the sections insensitive to the immunofluorescence techniques.

The indirect method, with its ability to include control sections, was preferable to the direct technique since it allowed the detection, and correct interpretation, of non-specific fluorescence.

Experiment 2: Development of immunoperoxidase techniques

The PAP technique was attempted and developed for the staining of CPV antigen.

Experimental Design

Initially, the PAP method described in the Materials and Methods section above was applied to frozen sections and sections fixed in 10% NBF, postfixed in corrosive formol and embedded in paraffin (Fig.28 - Method A); the appropriate controls were used.

Modifications were then applied to the initial PAP method. The first modification (Fig.28 - Method B) was to apply predigestion with trypsin (Difco, Michigan): sections were incubated with 1% aqueous trypsin solution at 37°C for periods

ranging from 5 minutes to 1 hour before staining. Again appropriate controls were used.

Secondly, a group of modifications were made (Fig.28 - Method C): the solvent for the trypsin solution was altered to a solution of Tris buffer pH 7.8 with 0.1% Calcium Chloride; the bridging antiserum was changed from the initial sheep anti-rabbit antiserum to a swine anti-rabbit antiserum known to have been used successfully in other PAP systems (O'Neil, personal communication); and the diluent for, and conditions of, incubation with the rabbit anti-CPV antiserum were altered.

Finally, using Method C, cardiac sections were stained using serial 1:10 dilutions of the six primary rabbit anti-CPV antisera (from undilute to 1 in 10^6) to determine the optimal working dilutions of the sera for the PAP method.

Results

The results are shown in Table 15. No staining of the intranuclear inclusions was obtained in the frozen and paraffin sections when Method A or Method B were used. With Method C, specific staining was present over the intranuclear inclusion bodies which were readily visible in the control tissue.

At the lowest dilution initially used, there was also considerable background staining. However, when the primary anti-CPV antisera were diluted and the optimal dilutions determined (Table 16), the optimal dilutions gave intense intranuclear staining (Fig.27) with minimal background reaction. There was good correlation between the optimal serum dilutions and the initial HAI titres of the sera.

Conclusions

Immunoperoxidase staining of CPV antigen could be achieved using a modified PAP technique on sections from tissues fixed in 10% NBF, post-fixed in corrosive formol and embedded in paraffin. The final successful technique is detailed in Table 17).

DISCUSSION

The decision to use myocardial tissue as the tissue of choice for developing immunocytochemical techniques proved to be well founded. This tissue provided a well localised site of known viral antigen which could be visualised by both light and electron microscopy with minimal background staining.

The easy development of the immunofluorescence techniques illustrates the main advantage of this method i.e. its simplicity. Only frozen tissue sections permitted specific staining, presumably due to complete preservation of antigenic reactivity in the section. The failure to demonstrate antigen in the tissues fixed in NBF and Bouin's fixative was almost certainly due to destruction of antigenic sites in the section by the cross-linking actions of these aldehyde fixatives. It may have been that enzymatic pre-treatment, similar to that subsequently applied in the PAP technique would have broken cross linkages, exposed antigenic sites and allowed specific immunofluorescent staining. It was disappointing that there was no specific staining following aldehyde fixation as this type of fixative has been used successfully in the fluorescence staining of immunoglobulin and Feline Leukaemia virus antigens in tissue sections (Sainte Marie, 1962; Dorsett and Ioachin, 1978). The reason for the failure of these agents to preserve antigenicity in the present studies is unknown. The alcohol fixatives preserve morphological detail by protein precipitation rather than cross-linking and precipitation of viral protein may have altered surface determinants in this system. Since cross-

linking is not involved, enzymatic pre-treatment of alcohol fixed tissue would be inappropriate.

The PAP technique has three overriding features. The first is its permanence: sections may be prepared, examined and stored for retrospective study. The second is that good histological definition is possible since formalin fixed material may be used and this greatly facilitates the identification of the cells containing antigen and its location. The first two features depend on the third, the sensitivity of this technique which enables the recognition in fixed tissues of antigenic sites which have been largely denatured.

This sensitivity is due to the absence of coupling agents which are required for conjugation in other immunocyto-chemical techniques. Coupling, using either high pH as in FITC conjugation, or by aldehyde agents, as in other, non-PAP, enzyme conjugation systems, can seriously impair the immunological reactivity of the antibody portion of such conjugates, thereby reducing their sensitivity. In the PAP system, the peroxidase is coupled to the antibody system by immunological means and not by damaging coupling agents, thereby optimising sensitivity.

Paradoxically, it was probably the sensitivity of the PAP technique which led to its failure to stain the intranuclear inclusions in fresh frozen tissues. This, almost certainly, was due to tight packing of specific anti-CPV antibodies over the antigen present on the section, since large numbers of antigenic sites would have been preserved by the minimal fixation procedures employed using the frozen tissue blocks. Such high density of specific antibody can lead to loss of the essential bifunctional reactivity of the bridging antibody (Sternberger, 1979), so that there is no adhesion of the bridging antibody to the PAP complex. Formalin fixation, by denaturation of the majority of the antigenic sites, leaves sparsely distributed antigen, resulting in less dense packing

of specific antibody and a return to the bifunctional reactivity of the bridging immunoglobulin. It was for this reason that after the initial work with PAP staining of frozen sections, by Method A, subsequent PAP staining by Methods B and C was limited to formalin fixed tissues.

However, in the present system, the formalin fixation appeared to denature CPV antigenic determinants to such an extent that the initial attempt to stain by PAP was still unsuccessful. Since the capsid of CPV is largely protein, it is likely that the effects of heat and organic solvents during processing were minimal and that the failure of specific staining was due to cross-linking of the antigen determinants by the aldehyde fixative. The eventual success of trypsin digestion, which breaks cross-links between protein molecules, would appear to confirm this.

The modifications made to the PAP staining technique to give Method C were suggested by Dr. Julia Polak of the Royal Postgraduate Medical School, Hammersmith (personal communication). The most important of these modifications was the buffering of the trypsin solution with the addition of calcium chloride to ensure that the pH and Ca^{++} concentration were at the optimal levels for effective trypsin action. The change of bridging immunoglobulin from commercial sheep anti-rabbit antiserum to a swine antiserum used successfully in other systems, simply removed a variable which might have been responsible for the failure of the technique. However, addition of BSA to the dilution fluid of the primary anti-CPV antiserum and increasing the incubation period of the primary antiserum with the sections have since been shown to be important features of the technique (Ward and Macartney, unpublished observations) although their mechanisms of action are unclear.

Having developed both immunofluorescence and immunoperoxidase techniques for the detection of CPV antigen, it was now possible to apply both procedures to the investigation

of the pathogenesis of CPV enteritis. The successful development of both techniques was a major advantage. The simplicity and rapidity of immunofluorescence made it ideal for the screening of a wide range of tissues from a substantial number of dogs; while the more complex immunoperoxidase procedure with its excellent morphological preservation could be used to aid the precise definition of antigen distribution and its relationship to the development of lesions.

The application of these techniques is described in the following Chapter 7.

TABLE 12.

DEVELOPMENT OF IMMUNOCYTOCHEMISTRY TECHNIQUES -
HAI TITRES OF INOCULATED RABBITS SIX DAYS AFTER
INTRAVENOUS BOOST

<u>Rabbit No.</u>	<u>HAI Titre</u>
1	65,000
2	8,192
3	1,040,000
4	32,768
5	32,768
6	130,000

TABLE 13.

DEVELOPMENT OF IMMUNOCYTOCHEMISTRY TECHNIQUES -
RESULTS OF IMMUNOFLUORESCENT EXAMINATION OF MYOCARDIAL
TISSUE BY DIRECT AND INDIRECT IMMUNOFLUORESCENCE.

<u>Fixative</u>	<u>Direct</u>	<u>Indirect</u>
10% NBF	-ve	-ve
Bouin's Fix.	±	-ve
Methanol	-ve	-ve
Ethanol	-ve	-ve
Fresh Frozen	+	+

TABLE 14.

DEVELOPMENT OF IMMUNOCYTOCHEMISTRY TECHNIQUES - RESULTS
OF INDIRECT IMMUNOFLUORESCENT STAINING OF MYOCARDIAL
TISSUE USING SERIAL DILUTIONS OF SPECIFIC ANTISERA.

<u>Rabbit</u>	<u>Optimal Dilution</u>	<u>HAI Titre</u>
1	1/100	65,000
2	1/10	8,192
3	1/1000	1,040,000
4	1/100	32,768
5	1/100	32,768
6	1/100	130,000

TABLE 15.

DEVELOPMENT OF IMMUNOCYTOCHEMISTRY TECHNIQUES - RESULTS
OF STAINING OF MYOCARDIAL TISSUE USING THE PAP TECHNIQUE.

PAP Method	Section Type	Result
Method A	Frozen	No specific staining
	Paraffin	No specific staining
Method B	Paraffin	No specific staining
Method C	Paraffin	Specific Intranuclear staining

TABLE 16.

DEVELOPMENT OF IMMUNOCYTOCHEMISTRY TECHNIQUES - RESULTS
OF STAINING OF MYOCARDIAL TISSUE WITH THE PAP TECHNIQUE
USING SERIAL DILUTIONS OF SPECIFIC ANTISERA

<u>Rabbit</u>	<u>Optimal PAP Dilution</u>	<u>HAI Titre</u>
1	1/1000	65,000
2	1/100	8,192
3	1/100,000	1,040,000
4	1/1000	32,768
5	1/1000	32,768
6	1/1000	130,000

TABLE 17.

DEVELOPMENT OF IMMUNOCYTOCHEMISTRY TECHNIQUES -
FINALISED METHOD FOR PAP STAINING.

1. Dewax sections, and remove mercuric pigment.
2. Wash sections in two changes of absolute alcohol, five minutes each.
3. Incubate sections with 0.5% hydrogen peroxide in methanol for 30 minutes at room temperature.
4. Rinse sections in Tris buffer pH 7.6, then in Tris buffer with 1% Normal Swine Serum, both for 10 minutes.
5. Immerse sections in a 1% trypsin solution in Tris buffer pH 7.8, with 0.01% calcium chloride for 30 minutes at 37°C.
6. Repeat Step 4.
7. Incubate sections with optimal predetermined dilution of primary antiserum in PBS pH 7.4 with 0.1% BSA and 0.1% sodium azide, for 18 hours at 4°C.
8. Repeat Step 4.
9. Incubate sections with swine anti-rabbit IgG (Mercia-Brocades, Weybridge) diluted 1:20 in Tris buffer, one hour at room temperature.
10. Repeat Step 4.
11. Incubate sections with peroxidase/anti-peroxidase raised in rabbit (Mercia-Brocades), diluted 1:20 in Tris buffer, one hour at room temperature.
12. Repeat Step 4.
13. Immerse sections in a solution prepared by dissolving 25 mg DAB in 50 ml Tris buffer, with 3% hydrogen peroxide, for 5 minutes at room temperature.
14. Wash sections with tap water.
15. Counterstain all sections with Meyer's Haematoxylin, and mount routinely.

Fig. 24: CPV Myocarditis - Control Tissue.

A basophilic intranuclear inclusion body may be seen in a cardiac myocyte. The inclusion is surrounded by a clear halo. Nuclear chromatin is margined.

H & E x 400.

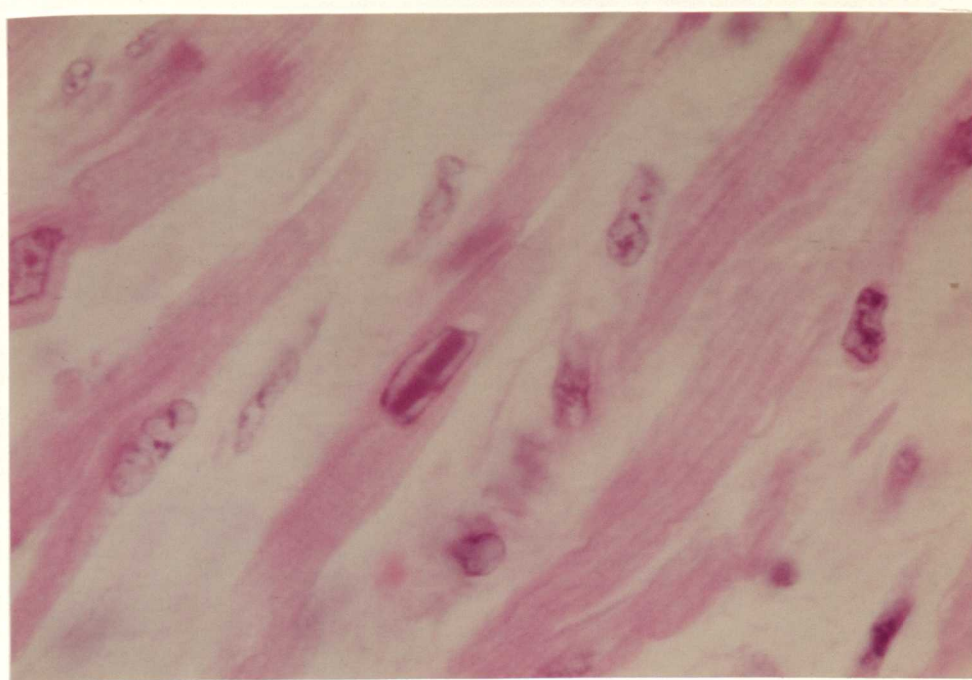


Fig. 25: CPV Myocarditis - Control Tissue.

There is margination of nuclear chromatin to the nuclear membrane (small arrows). A large aggregate of CPV virions and perichromatin granules may be seen (large arrows).

Transmission Electron Microscopy x 40,000.

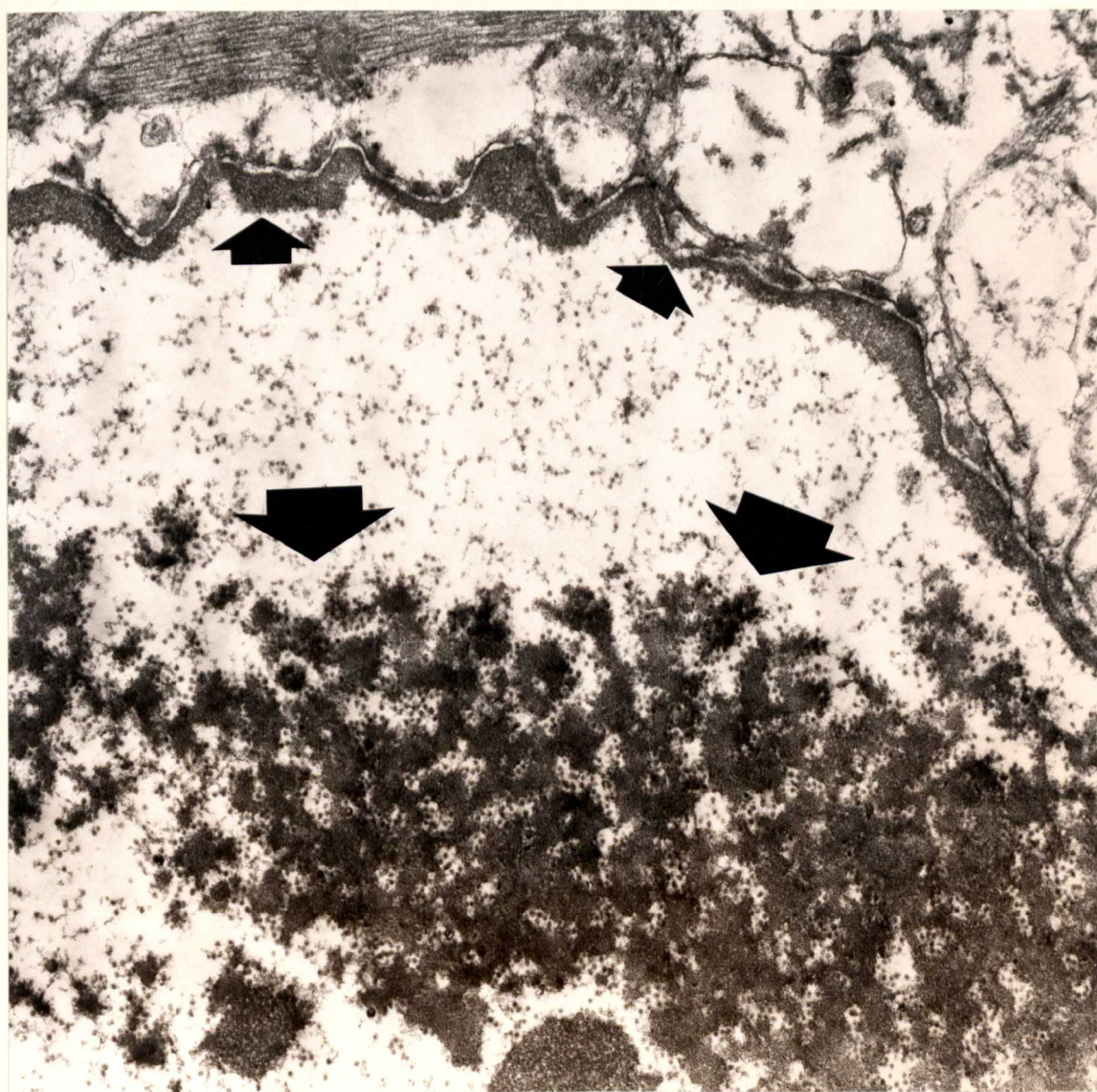


Fig. 26: CPV Myocarditis - Immunofluorescent Staining.

Note the intense staining over intranuclear inclusion bodies, some of which are cut in cross section.

Immunofluorescent staining x 250.



Fig. 27: CPV Myocarditis - Immunoperoxidase Staining.

There is intense intranuclear staining in the myocyte containing an intranuclear inclusion body.

Immunoperoxidase staining x 400.

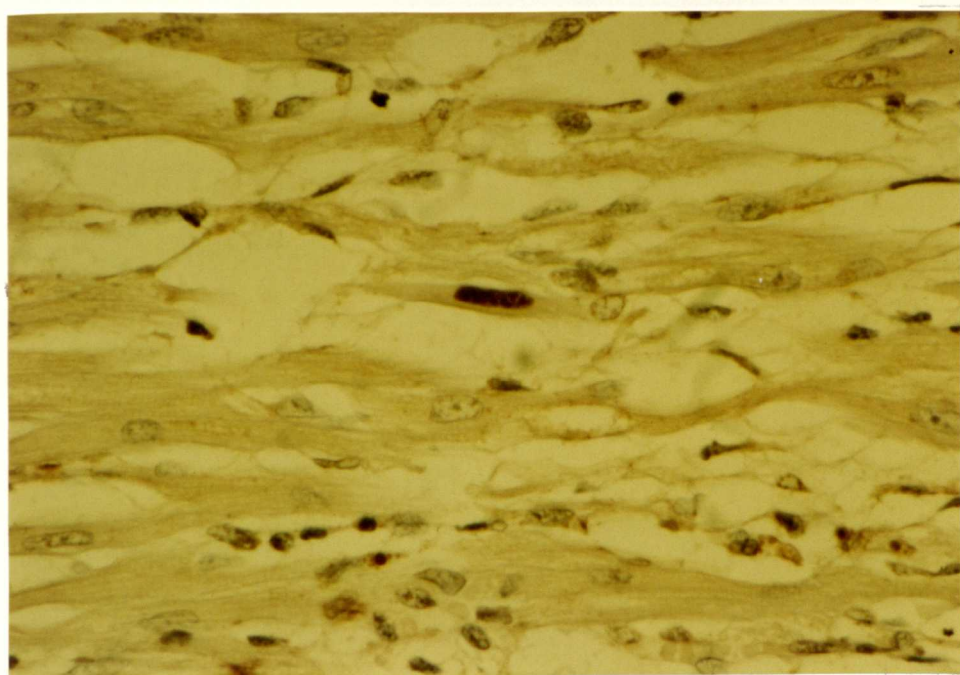
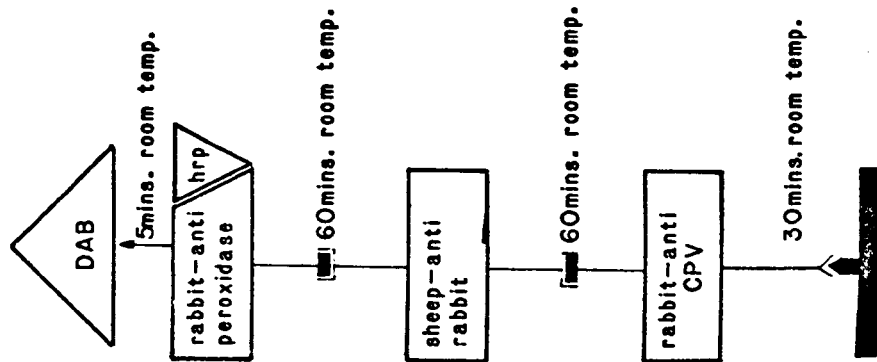


Fig. 28.

Development of immunocytochemistry techniques -
Methods used in development of immunoperoxidase technique.

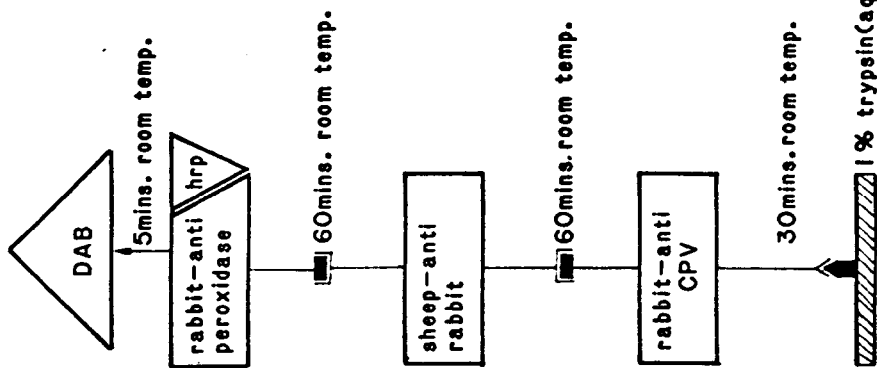
METHOD A

Fixed sections: No Staining
Frozen sections: No Staining



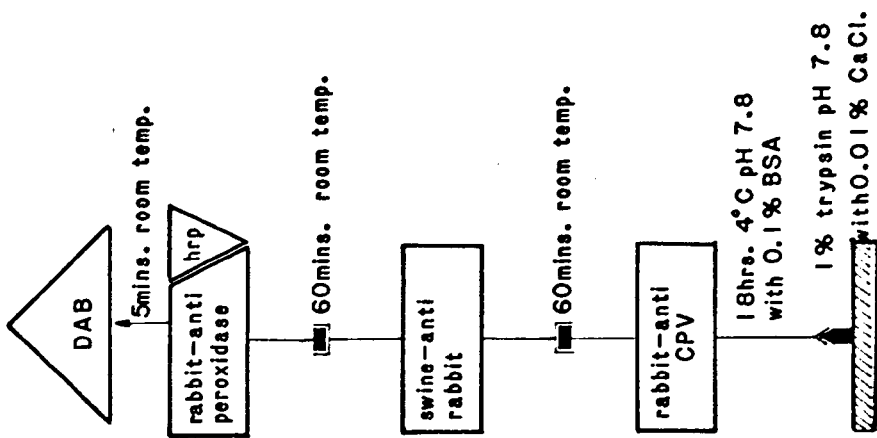
METHOD B

Fixed sections: No Staining



METHOD C

Fixed sections: Intra-nuclear staining



CHAPTER 6 : ORAL INFECTION OF DOGS WITH CPV OF FAECAL ORIGIN
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INTRODUCTION

As the basic parameters of experimental CPV infection had been established with tissue culture derived virus as described in Chapter 3, the decision was made to study the clinical findings and pathological changes following oral infection with high titre purified CPV of faecal origin.

The original intention was to describe the sequential pathological changes and, in particular, to demonstrate the distribution of antigen during the early stages of the disease. However, the schedule of the experiment was disrupted by a major fire at the laboratory the day after infection, which did not affect the dog accommodation but made sampling and killing of dogs in the first days after infection impossible and limited the type of material which could be processed from dogs killed later in the study. In particular, the immunocytochemical investigations had to be abandoned and these were subsequently undertaken in the study described in Section 7.

MATERIALS AND METHODS

Experimental Animals

The source, housing and maintenance of experimental animals has been described (Chapter 2). For this experiment, 14 pups, eight weeks old, were used.

Experimental Design

On arrival at the laboratory, the dogs were randomly divided into two groups (Table 18). The first group, of two dogs (11 and 12), was killed at the start of the experiment to monitor the status of the dogs and to provide control tissue. The remaining 12 dogs (13-24) were infected by oral administration of 2 ml each of purified virus of faecal origin.

The purification method has been described (Chapter 4); the virus had an HA titre of 130,000 per 0.025 ml, with an infectivity titre of $10.5 \log_{10}$ TCID₅₀/ml.

After infection, dogs were housed separately to permit collection of individual faecal specimens. Each dog was examined clinically each day and blood samples for haematological and serological investigations were collected by jugular venipuncture. Rectal temperatures were recorded on the day prior to infection and on the day of infection, but not thereafter. Dogs were killed at 5, 6, 7, 12 and 13 DPI. Three dogs died, one at 6 DPI and two at 7 DPI. A full post-mortem examination was carried out on each animal, the total body weight and thymic weights noted and tissues taken into 10% NBF and Bouin's fixative as described in Chapter 2.

Procedures Employed

Clinical and post-mortem examinations, haematological, serological and histological procedures and detection of virus excretion were carried out as described in Chapter 2.

RESULTS

Control Dogs

The control dogs killed at the start of the experiment were clinically healthy. Rectal temperatures were within the normal range, as were the values obtained from haematological examination which are recorded in Tables 20, 21 and 22. Viral haemagglutinins were not detected in faecal samples from these animals and antibody to CPV was not detected in their sera.

Post-mortem examination revealed no macroscopic abnormalities. The thymic weights are recorded in Table 28 and

are also expressed in the form of a ratio to the total body weight. In both dogs, the thymus appeared as a pale, pink, lobulated organ which almost completely filled the anterior mediastinum.

Histological examination revealed no underlying pathological lesions. In the lymph nodes of both dogs, large, densely populated germinal follicles were evident (Fig. 40). The lobules of the thymus were separated by a delicate connective tissue stroma. Each lobule was clearly divisible into a cortex and medulla (Fig. 37). The predominant cell type in both areas was the typical small dark lymphocyte with a high nuclear/cytoplasmic ratio. These cells were densely packed in the cortex but less so in the medulla. A full complement of erythroid and myeloid precursor cells was evident in the bone marrow along with numerous mature neutrophils (Fig. 50).

Well defined crypts and villi were observed at all levels of the small intestine. Numerous mitotic figures could be observed in the crypt epithelium throughout the small intestine. In addition, individual small dark amphophilic cells were occasionally observed in the epithelium. The villi in the duodenum were tall and slender with few goblet cells in their columnar epithelial lining. Distally, the villi were shorter and goblet cells were more numerous (Fig. 44). At all levels of the small intestine, the epithelial lining had a well defined microvillous brush border. Prominent germinal follicles were present in the GALT throughout the alimentary tract. The GALT was particularly prominent in the ileum and in the associated mucosa the villi were shortened and thick with distorted and irregular crypts.

Infected Dogs

Clinical Signs

The clinical findings are summarised in Table 19.

Clinical signs were first observed at 4 DPI, when dogs 13, 14 and 17 were slightly dull (Fig. 29) and refused solid food although their fluid intake was maintained; the other dogs appeared clinically normal. By 5 DPI, all 12 infected dogs were dull and most were anorexic. Dogs 13 and 14 were most severely affected, refusing both solid food and fluid: both vomited every 15-40 minutes, producing small amounts of clear or frothy mucus which was occasionally yellow due to bile staining. Both dogs also had diarrhoea: in the morning, stools were soft with a mucous coating but during the day this changed and, by noon, the faeces were liquid and were passed without apparent effort or straining. The faeces were dark red in colour with a particularly unpleasant foetid odour, akin to that of rotting vegetation. The animals became severely dehydrated (Fig. 30) and progressively duller during the afternoon; they finally became prostrate and were killed in extremis late in the afternoon of 5 DPI.

Of the 10 dogs remaining on the evening of 5 DPI, dog 18 was severely ill. This pup had started to vomit at about mid-day and continued to vomit at approximately 30 minute intervals thereafter, producing material similar to that of dogs 13 and 14. No faeces were passed by dog 18 until the evening of 5 DPI when the stool was soft in consistency with mucus on the surface. The pup was very dull and dehydrated and was found dead on the morning of 6 DPI.

At this time, another two dogs (15 and 19) were extremely ill and were killed in extremis. Dogs 15 and 19 had been dull at 5 DPI although neither vomiting nor diarrhoea had been noted. However, on the morning of 6 DPI, both were prostrate and passing foul, foetid, fluid faeces, the colour of cherry brandy (Fig. 31).

Two further dogs, 16 and 17, deteriorated rapidly during 6 DPI with soft mucoid faeces in the morning which progressed through watery dark brown fluid to bright red

dysenteric material containing shreds of mucosa by later afternoon. The animals were dehydrated, stood with arched backs and resented abdominal palpation. Dog 16 vomited at 90 minute intervals throughout the day but no vomition was observed in dog 19. Both dogs were killed in the late afternoon.

Of the five dogs remaining late on 6 DPI, two dogs (20 and 22) had profuse fluid diarrhoea with, in dog 20, occasional vomiting. Dogs 21, 23 and 24 had soft, semi-formed faeces with flecks of mucus.

On the morning of 7 DPI, dogs 20 and 22 were dead; pools of dysenteric faeces with mucosal shreds were present on the floors of their pens. During 7 DPI, dog 21 vomited frequently and passed dark red, foul faeces. There was severe dehydration, abdominal pain and prostration and the dog was killed early in the afternoon.

Dogs 23 and 24 were never severely affected after being dull at 5 and 6 DPI with occasional vomition on 6 DPI. They were killed, having shown no further clinical signs, at 12 and 13 DPI respectively.

Haematological Findings

Dogs were sampled on the day before infection, the day of infection, in most cases at 5, 6 and 7 DPI and at intervals thereafter. The absolute total leukocyte counts and the differential counts of neutrophils and lymphocytes are shown in Tables 20, 21 and 22.

Pre-infection, the total leukocyte counts of all dogs were within the normal range, although there was quite wide variation ($11.4 - 19.7 \times 10^9/l$). The absolute neutrophil and lymphocyte counts also varied widely ($7.4 - 14.7$ and $3.3 - 7.9 \times 10^9/l$ respectively) but were again within the normal range.

There was, in most dogs, a drop in total circulating leukocytes at 5 DPI compared to the pre-infection levels although the values found (as low as $7 \times 10^9/l$) were still just within the normal range. This relative reduction in total circulating leukocyte counts was due largely to a reduction in absolute lymphocyte counts, with two dogs (14 and 19) having lymphocyte counts below the normal range (0.525 and $0.5 \times 10^9/l$ respectively), thereby indicating an absolute lymphopaenia in these animals. There was also some reduction in the numbers of circulating neutrophils at 5 DPI in 7 of the 11 dogs sampled while the remaining four dogs showed a rise. No dog on this day had absolute neutrophil counts below the lower limit of normality.

On 6 DPI, there was an absolute leukopaenia in all dogs sampled, the lowest total count being $1.1 \times 10^9/l$. The numbers of circulating lymphocytes in these dogs were similar to those noted at 5 DPI and the leukopaenia was associated with a very marked reduction in the absolute neutrophil counts in all dogs. Thus, at 6 DPI, there was a panleukopaenia, with a relative lymphopaenia (in all but dog 19 where the lymphopaenia was absolute), and an absolute neutropaenia. An additional finding at 6 DPI was the presence of numerous activated lymphocytes in the peripheral blood of dog 15 (Fig. 32).

In dog 21, at 7 DPI, the total leukocyte count was reduced compared to the pre-infection levels in this dog. This 7 DPI level ($10.2 \times 10^9/l$) was nonetheless within the normal range even in the face of an absolute neutropaenia ($2.85 \times 10^9/l$); this was due to a high total lymphocyte count ($6.01 \times 10^9/l$). Numerous activated lymphocytes were noted in this blood smear and may in part explain the absolute lymphocytosis.

At 9 DPI, in dogs 23 and 24, there was an absolute leukocytosis. In both animals the total circulating lymphocyte counts were high (11.61 and $12.13 \times 10^9/litre$ respectively), and this absolute lymphocytosis was again associated with numerous

activated lymphocytes in the blood smears. In addition there was an absolute neutrophilia in dog 24 at this time. In dog 23 at 12 DPI, all haematological parameters were within the normal range. However at 13 DPI there was a leukocytosis due to both an absolute lymphocytosis and neutrophilia in dog 24.

Viral Excretion

The dogs had been individually penned to facilitate the collection of faecal specimens. However, stools were not produced daily by each animal and so faecal swabs were taken in addition. Swabs were taken on the day of infection and at 5 and 6 DPI. Dogs 20 and 22 were swabbed at 7 DPI and remaining dogs were swabbed at intervals thereafter. Faecal samples were collected and intestinal contents were collected at post-mortem examination. Swabs and faecal and intestinal material were subjected to the HA test.

Viral haemagglutinin, at a titre of 64, was present in the only faecal sample obtained at 4 DPI, from dog 20, (Table 23). Specific haemagglutinin was detected in all the faecal specimens produced at 5 DPI, with titres ranging from 16 to 2048. Haemagglutinin was again detected in all the faecal samples collected at 6 DPI. There were titres of 512 - 1024 in two of the four faecal specimens (from 20 and 22) collected at 7 DPI while the other samples were negative. Small amounts of viral haemagglutinin were detected in the two faecal samples obtained from dog 23 at 8 and 9 DPI. The results obtained from faecal swabs (Table 24) correlated closely with the results from faecal specimens; in some, the swab titres were higher.

Viral haemagglutinin was detected in samples of excreted faeces and intestinal content of both dogs killed at 5 DPI. It was also found in the excreted faeces of all but one of the dogs which died or were killed at 6 and 7 DPI, although it was apparently absent from the intestinal content of the same animals (Table 23).

Serological Findings

The results of examination of sera for antibody to CPV are shown in Table 25. Antibody was not detected in sera taken prior to infection but when sampling was resumed at 5 DPI, antibody was found in all but four of the dogs with titres ranging from 8 to 256. By 6 DPI, the three surviving dogs which had been seronegative at 5 DPI had also developed antibody at titres of 16 to 1024. All dogs which had been seropositive at 5 DPI showed at least a further fourfold rise in antibody titre by 6 DPI and titres of 1024 or greater were found in all sera collected on or after 7 DPI.

Specific antibody to CPV was detected in the intestinal content of dogs examined at 6 and 7 DPI (Table 26), the intestinal titre being lower than the corresponding serum titre. Antibody was not detected in the intestinal content of either dog at 5 DPI or of the dogs killed at 12 and 13 DPI.

Pathological Findings

Macroscopical Findings

In this study, as in the earlier experiment described in Chapter 3, changes in the lymph nodes and thymus were again a major feature (Table 27). In addition, and in contrast to the earlier investigation, severe lesions were found in the alimentary tract of the majority of dogs in this study.

In dogs 13 and 14 (killed at 5 DPI) and dog 15 (killed at 6 DPI), changes were most pronounced in the duodenum with decreasing severity distally. In these animals, the duodenal wall was thickened and rigid with an oedematous, flattened mucosa; the lumen was narrow and contained scanty amounts of yellow-brown, mucoid fluid (Fig. 33). The serosal surface was congested. Similar, though less severe changes were present in the coils of the jejunum and the ileum was also thickened and

slightly oedematous. The stomach contained large quantities of thick "egg-white" mucus but the underlying gastric mucosa appeared normal (Fig. 34).

In dogs 16, 17, 18 and 19, the other dogs examined at 6 DPI, similar intestinal changes were observed but the jejunum, rather than the duodenum, was most severely affected with marked serosal congestion, rigidity of the intestinal coils and thin, blood-stained fluid contents. In the ileum, changes again consisted of slight thickening of the wall with mucosal oedema and some petechiation of the mucosal surface. Large quantities of mucus were again observed in the gastric lumen of these dogs, although the wall and mucosa of the stomach were apparently normal.

The dogs which were killed in extremis and examined immediately at 5 and 6 DPI did not show the characteristic 'salt-glaze' granularity of the serosal surface which is frequently seen at post-mortem examination of field cases of CPV enteritis. In contrast, dog 18, which died overnight and was not examined until a few hours after death, did exhibit this feature.

Congestion, thickening and rigidity of the wall of the duodenum, jejunum, and, to a lesser extent, the ileum, were noted again in three dogs examined at 7 DPI. Once more the lumen appeared narrow, with a flat mucosa covered by scanty blood-stained content. Serosal granularity was again noted in the two dogs which had died during the night but was not observed in dog 21, which was killed and examined immediately after death.

In dogs 23 and 24, killed at 12 and 13 DPI, the wall of the duodenum and jejunum was slightly thicker than in control dogs but there was no congestion or rigidity of the wall or narrowing of the lumen. The mucosa had its typical velvety appearance and the content was normal.

The other striking findings in the alimentary tract of infected dogs were changes associated with the GALT. In animals examined at 5, 6 and 7 DPI, during the phase of clinical illness, GALT was very noticeable on the generally congested serosal surface of the intestine as pale, sharply demarcated areas 0.5 - 1 cm in diameter. On the mucosal surface, the GALT appeared as well-defined, depressed circles or ovals, about 2 mm deep with a congested or even haemorrhagic surface (Fig. 35). The surrounding mucosa appeared to "overhang" the depressed GALT. In dogs 23 and 24, killed at 12 and 13 DPI, the GALT was still noticeable as pale areas up to 2 cm diameter through the serosa with a bulging, 'fleshy, appearance on the mucosal surface.

There were changes in the lymphoid system of all infected animals, with the most striking changes observed in the thymus, although peripheral lymph nodes and spleen were also affected.

In the dogs killed at 5 and 6 DPI, the thymus was smaller than in controls and was slightly gelatinous in appearance (Fig. 36). The body/thymic weight ratios are shown in Table 28. The ratio was noticeably higher in the dogs killed at 5 and 6 DPI confirming the reduction in thymic mass. The thymus was again smaller than normal at 7 DPI, although it was no longer gelatinous; again, the body/thymic weight ratio was greater than in controls. The thymus in the dogs killed at 12 and 13 DPI was reduced to a thin, lace-like structure; it was composed of small pale islands of lymphoid tissue scattered in sparse connective tissue stroma. There was a very large increase in the thymic/body weight ratios for these two dogs.

Both the visceral and carcass lymph nodes of dogs examined at 5, 6 and 7 DPI were oedematous. The cut surface did not bulge upon sectioning and it was difficult to determine a delineation between cortex and medulla. In those animals which had died, the spleen had a mottled red/purple colouration.

In the dogs killed at 12 and 13 DPI, the peripheral lymph nodes were enlarged and reactive with a sharp demarcation between a bulging nodular cortex and the medulla. The splenic white pulp was more obvious than in control dogs, being visible as white areas 0.25 - 0.5 cm in diameter on the cut surface of the spleen.

Microscopical Findings

All tissues detailed in Chapter 2 were examined histologically. Changes were found in the thymus, peripheral and visceral lymph nodes and intestine of all the infected dogs. In some animals,

changes were also found in the bone marrow. In one dog, dog 16, there were individual pyknotic hepatocytes in a perilobular position. Pathological changes were not found in other organs. To facilitate detailed histological descriptions, the changes will be described for each organ affected.

Thymus

In dogs 13 and 14, killed at 5 DPI, the lobules of the thymus were separated by oedematous connective tissue stroma. In general, the cortex was depleted of thymocytes compared to that of controls. Large amounts of cellular debris were scattered through the cortex with small aggregates of dark staining, pyknotic thymocytes with some karyorrhexis and fragmentation of dead cells. The medulla was less affected.

In the dogs examined at 6 DPI, the predominant feature was severe depletion of the thymic cortex. This was so extensive that the medulla stained more darkly than the cortex, a reversal of the normal pattern. In the cortex itself, there was little active lymphocytolysis with only scattered foci of pyknotic cells. The medulla was also depleted although to a

lesser extent than the cortex. The interlobular connective tissue was oedematous but less so than at 5 DPI.

Similar changes were present in the dogs examined at 7 DPI (Fig. 38). Active lymphocytolysis was not a feature and interlobular oedema was even less marked.

In the thymus of the dogs killed at 12 and 13 DPI, the cortex was still hypocellular and there appeared to be an increased amount of intercellular ground substance. Foci of darkly staining, thymocytes with numerous mitotic figures were present scattered throughout the cortex. The medulla in these animals was more densely populated than in control sections and because of this the staining pattern was again reversed (Fig.39). Interlobular oedema was not a feature but the lobules of lymphoid tissue were widely separated by dense connective tissue.

Carcase and Visceral Lymph Nodes

In dogs 13 and 14, killed at 5 DPI, the germinal follicles were depleted of centrocytes, and numerous "histiocytic" cells could be seen in the germinal centres. In some follicles there were also pyknotic cells with karyorrhexis and fragmentation (Fig. 41). The paracortex was slightly hypocellular especially in dog 13, where numerous pyknotic lymphocytes were scattered throughout this area. In dog 13, neutrophils were present in the germinal centres of some nodes (Fig. 42).

There was no evidence of active lymphocytolysis in the dogs examined at 6 DPI. In these dogs, the germinal follicles were hypocellular and the centres were filled by the histiocytic cells. The paracortex was depleted in four of the five dogs examined, the exception being dog 16 in which there was evidence of early paracortical expansion with large basophilic 'blast' type cells. These cells had a lower nuclear/cytoplasmic ratio

and a more open nucleus than the typical small lymphocyte. In addition, in dog 16, increased numbers of plasma cells were found in the medullary cords.

In dogs 20 and 22, which died during the night of 6 DPI, the germinal centres were composed mainly of histiocytic cells with residual lymphocytolysis in the germinal centres and paracortex. There was no evidence of lymphoid regeneration or repopulation. In contrast, in dog 21, killed at 7 DPI, there was marked expansion of the paracortex. No lymphocytolysis was present and there was repopulation of the depleted germinal follicles by centrocytes and centroblasts.

In all nodes of the two dogs killed at 12 and 13 DPI there were marked regenerative changes with expansion of the cortex and paracortex. In the cortex, the germinal follicles were reactive. There was an increased population of fixed sinusoidal macrophages and large numbers of plasma cells packed the medullary cords.

Spleen

In the dogs examined at 5 and 6 DPI, there was active lymphocytolysis with pyknosis and karyorrhexis in the white pulp. In dogs examined at 7 DPI, lymphocytolysis was not marked and the white pulp was not prominent. In the dogs killed at 12 and 13 DPI, in contrast, there was expansion of the white pulp which contained active germinal follicles.

Alimentary tract

Lesions were found in the alimentary tract of all infected dogs with the small intestine predominantly affected. No changes were present in the gastric mucosa.

In the small intestine, the basic lesion appeared to affect the crypt/villus unit. The extent and severity of the change varied between individual crypt/villus units in the same animal and with time after infection between animals. Four basic patterns of crypt/villus lesion were identified and these patterns are illustrated in Fig. 43.

The first pattern, pattern A, consisted of relatively minor changes from normal crypt/villus architecture. Numerous small dark amphophilic cells were present in the proliferative area of the crypt epithelium immediately above basal crypt cells. The associated villi appeared reduced in height but there was no fusion of the villi which were covered by cuboidal cells increasing to columnar in height towards the tips; the microvillus brush border appeared to be intact. There was no increase in cellularity of the lamina propria and congestion was not a feature.

In pattern B, more dramatic alterations in crypt/villus architecture were apparent. The crypts were lined by attenuated, flattened epithelial cells in some of which palely amphophilic intranuclear inclusions could be seen (Fig. 45). In some instances, the lumina of affected crypts were dilated (Fig. 46). The basal crypt cells were unaltered but the villi were commonly absent or, where present, grossly stunted. Where villi were discernable, their epithelium was thin, lacked a brush border and was often separated from the underlying lamina propria. In those cases where there was complete atrophy of villi, the superficial epithelium was often totally lost. A mild neutrophil infiltrate was occasionally seen in the lamina propria but large numbers of neutrophils were generally present in a thick mucoid exudate consistently found on the mucosal surface. There was variable congestion of the lamina propria and when the crypt/villus lesions were confluent there was oedema of the submucosa and muscularis. The presence of oedema dramatically altered staining of the connective tissue stroma which was then intensely eosinophilic and the nerve plexuses became extremely prominent.

In pattern C, there was almost total obliteration of the normal intestinal architecture. There was complete atrophy of villi with collapse of the remaining mucosa and subsequent loss of normal crypt structure. The mucosa consisted of a homogeneous mass of cells. Remnants of crypt structure could sometimes be discerned only by the connective tissue pattern in the lamina propria since in many instances there were no clearly recognisable epithelial cells. Dilated crypt remnants, lined by attenuated epithelial cells, were also present. In some dilated crypts, large epithelial cells with abundant pale eosinophilic cytoplasm and pale open nuclei with prominent nucleoli were found; three or four nuclei were sometimes present in a single cell (Fig. 47). Where this pattern was observed, there was often loss of surface epithelium and in some areas large numbers of neutrophils were present in an overlying mucoid exudate. The lamina propria was congested and when lesions were confluent there was, as in pattern B, oedema of the submucosa and muscularis.

In pattern D, there was regeneration of crypt architecture. The epithelial lining of the crypts was cuboidal with a darkly basophilic cytoplasm. The nuclei of these cells were basal in position and numerous mitotic figures could be observed. This type of epithelium was present towards the base of all the crypts and in some crypts merged with an attenuated epithelium, whereas in others it lined the entire crypt length. Villi were present ranging in height but were generally shorter than in control dogs (Fig. 48). The surface epithelium ranged from an attenuated layer over the partially lined crypts to a low cuboidal layer over those crypts lined completely with regenerating epithelium. There was an apparent increase in the number of goblet cells, both within the crypts and in the epithelium over the villi. Congestion and oedema of the lamina propria and submucosa was present but was less marked than in patterns B and C. Cellular infiltration was not a feature.

The extent of any given pattern of lesion varied from focal through to confluent. This extent was graded on a "+" to "+++" scale and the summary of the patterns and extent of the lesions present at different levels of the small intestine is given in Table 29.

In the two dogs killed at 5 DPI, extensive lesions were found in the duodenum, with less marked changes in the distal small intestine. Lesions were more extensive in dog 13 than in dog 14. Crypt/villus lesions of pattern B were present in the duodenum whereas the more minor changes of pattern A were present in the ileum. There was haemorrhage onto the mucosal surface of the duodenum and jejunum, with congestion of the serosa in both animals.

There was marked destruction of the lymphoid elements of the GALT in both dogs 13 and 14 with pyknosis and fragmentation of lymphoid cells; in some follicles there was frank coagulative necrosis. In crypts over the GALT, there were some amphophilic cells, together with some attenuation of crypt epithelial cells. However, in view of the normally bizarre mucosal morphology in these areas, it was not possible to assess the degree of villus stunting.

In the caecum and colon of both dogs, there was dilation of some of the mucosal glands, with an increase in the numbers of small dark amphophilic cells in the proliferative epithelium. Many of these small dark cells appeared to have been extruded into the lumen of the dilated glands, with the remainder unaffected.

In dogs 15 to 19, examined at 6 DPI, lesions of pattern B were seen at all levels of the small intestine. Within the range of this pattern, crypt dilation was more marked than at 5 DPI. In addition, the distribution of crypt/villus lesions was more extensive and totally confluent in the jejunum.

The classification of the ileal lesion presented some difficulties. In those areas of the ileal mucosa outwith lymphoid areas, focal areas were affected by the pattern B lesions. There was also epithelial damage in crypts overlying the GALT, evinced by epithelial attenuation and dilation of the crypt lumina. However, it was difficult to estimate the extent of these lesions since mucosal morphology over these areas is normally bizarre. In addition, as at 5 DPI, there was marked lymphoid depletion and necrosis in the germinal centres of the GALT and consequently it was unclear to what extent disruption of the underlying lymphoid tissues contributed to any alteration in mucosal morphology. Changes similar to those seen in the dogs killed at 5 DPI were found in the caecum and colon of these animals.

Confluent lesions of patterns B and C were seen throughout the small intestine of the dogs examined at 7 DPI. In dog 21, the changes were most extensive in the duodenum, but interestingly in this area a few crypts were noted with a darkly basophilic cuboidal epithelium which suggested an early attempt at regeneration (Fig. 49).

In those dogs found dead at 7 DPI there was very marked dilation of the serosal lymphatic vessels, and this possibly accounted for the 'salt glaze' serosa seen at post-mortem examination. This dilation was not a feature of the dog which was killed at 7 DPI. The changes in the GALT of dog 21 and in the caecum and colon of all three dogs were similar to those seen in the dogs killed at 6 DPI. Extensive necrosis was observed in the GALT of dogs 20 and 21 which had died.

In all areas of the small intestine of the dogs examined at 12 and 13 DPI changes of pattern D were present. The GALT was expanded: large numbers of germinal follicles were present.

No changes were found in the caecum or colon of these dogs except for expansion of the GALT.

Bone Marrow

In the animals examined at 5, 6 and 7 DPI, there were fewer mature neutrophils in the bone marrow when compared to control sections. Blast cells were more prominent and cellular destruction was not a feature, with only a few pyknotic or karyorrhexic cells evident. Intranuclear inclusion bodies were observed in two dogs. In dog 13, killed at 5 DPI, there were pale amphophilic intranuclear inclusion bodies in large blast cells, the inclusions being sparse throughout the marrow (Fig.51). Occasional intranuclear inclusion bodies were found in the marrow of dog 15, killed at 6 DPI, although in this animal they were not as widespread. There were no abnormalities noted in the marrows of the dogs killed at 12 and 13 DPI when a full complement of mature neutrophils was present. Blast cell activity in these specimens was comparable to that observed in control sections.

Bouin's Fixed Tissues

The sections prepared from tissues fixed in Bouin's revealed identical lesions to those described above. The only difference was that inclusions and the small amphophilic cells present in the crypts were more immediately obvious.

DISCUSSION

The aim of this experiment was to study the effect of oral challenge with high titre purified CPV of faecal origin. When compared with the results of the preliminary study using infection with tissue culture derived virus, the most dramatic difference was the development of severe enteric disease in the infected dogs. Three of the infected animals died, seven became so ill that they had to be killed in extremis and only two recovered.

The experimental disease was an acute illness with a sudden onset and a short course. Severe illness started at 5 DPI and deaths were recorded at 6 and 7 DPI. Surviving dogs were clinically normal by 7-8 DPI.

The first clinical signs were dullness and anorexia followed by the onset of vomiting and diarrhoea. The time from the onset of dullness to death was as short as 48 hours and it was apparent that rapid decline followed by prostration could occur within 8 hours of the start of enteric signs. All of the dogs developed diarrhoea which varied in consistency from soft faeces coated with mucus to frank dysentery. Vomiting was less consistent both in its appearance and in its frequency in individual dogs. It was, moreover, difficult to record accurately since in most cases the vomitus was a clear fluid which was easily absorbed by bedding or even by the dogs' coats.

No other workers have reported a similar severe disease with fatalities following oral infection with CPV of faecal origin. Azetaka et al. (1981) observed fatal disease in one dog infected orally with virus of tissue culture origin (5th passage in CRFK), while Robinson et al. (1980b) were able to reproduce severe clinical disease in a litter of five, 7-week old puppies following intravenous infection with tissue culture derived virus isolated from a case of CPV myocarditis.

In view of the successful reproduction of severe clinical disease, closely mimicing the descriptions of natural field outbreaks, it was particularly disappointing that the laboratory fire prevented the sequential haematological examination originally planned. Nonetheless, several interesting haematological observations were still possible. The method of collection of blood samples in this study, i.e. by jugular venepuncture, did, as expected, prove to be more reliable than the ear vein technique used in the preliminary study: in the present experiment, all pre-infection samples were within the normal range and there was considerably less variation than had been obtained with the previous technique.

In the infected dogs at the peak of clinical illness, there was a relative lymphopaenia and an absolute neutropaenia (panleukopaenia) in the moribund animals. The lymphopaenia may be relatively simply explained by the marked destruction of the lymphoid tissues throughout the body. The explanation for the neutropaenia in the moribund dogs would initially appear to be less obvious, as there was little apparent damage to myeloid precursor cells in the marrow although intranuclear inclusion bodies were observed in the dogs. There was, however, severe depletion of mature neutrophils in the marrow of these severely affected animals and a large number of neutrophils were observed in the mucus overlying the extensive intestinal lesions. It therefore seems possible that the neutropaenia was due to sudden migration and loss of circulating neutrophils through the damaged intestinal epithelium. In support of this hypothesis, it is notable that in other studies neutropaenia has only been described in association with severe enteric disease (Azetaka et al., 1981); Robinson et al., 1980b). Where only mild or minimal clinical disease has been produced (Robinson et al., 1980b; Carmichael et al., 1981) only a reduction in circulating lymphocytes has been described, without neutropaenia. It would appear that the panleukopaenia of clinical CPV enteritis is due to a combination of failure of lymphocyte production together with a loss of circulating neutrophils.

The collection of faecal samples for the assay of viral haemagglutinin and blood samples for serological examination was also disrupted by the laboratory fire and the results for these are also incomplete. Haemagglutinin was detected in the faeces of affected dogs from 5 to 9 DPI, but the highest titres were recorded at 5, 6 and 7 DPI. The results obtained from examination of rectal swabs correlated well with those obtained from faecal specimens, but it was noticeable that in this experiment, where the dogs were diarrhoeic, faecal material was consistently present on the swabs, in contrast to the preliminary study where little faecal material was found on swabs from non-diarrhoeic dogs. However, as it is easier to quantify whole faecal specimens than faeces on swabs, it was considered that faecal specimens rather than rectal swabs were the most reliable base material.

The intestinal contents were also assayed for the presence of haemagglutinin. Haemagglutinin was detected in both faeces and intestinal contents of dogs killed at 5 DPI. In dogs examined at 6 and 7 DPI the intestinal contents were found to be negative although there was detectable haemagglutinin in their faeces collected earlier on the day of death. In the dogs killed at 12 and 13 DPI, both faeces and intestinal contents were negative. The explanation for this may lie in the results obtained from antibody analysis of serum and intestinal contents.

Serum antibody to CPV was detected in some of the dogs at 5 DPI and in all of the samples thereafter, a pattern similar to that found in the preliminary experiment. Antibody to CPV was also detected in the intestinal contents of the dogs examined at 6 and 7 DPI, but not in the contents of the dogs killed at 5 DPI nor in either of the two recovered dogs examined at 12 and 13 DPI.

In view of the extensive mucosal damage present in the dogs examined at 6 and 7 DPI, the origin of the intestinal

antibody found at this time seems likely to be a direct leak from the circulation. Antibody present in the intestinal lumen would be likely to complex any virus in the lumen which would result in a negative result on haemagglutinin assay of intestinal contents. In the dogs examined at 5 DPI, although there was intestinal damage, only low serum antibody levels were present so that failure to detect antibody in the contents at this time is not surprising. Similarly, in the dogs examined at 12 and 13 DPI, although serum antibody was high, detection of an intestinal leak would not be expected in the absence of severe intestinal damage. The development of a leak of serum into the intestine must occur over a short period of time since, in the dogs killed at 6 and 7 DPI, viral haemagglutinin was not detected in the intestinal contents although it had been present in faecal material collected only a few hours earlier.

The severe clinical picture was mirrored in the pathological changes encountered in this experiment with lesions consistently present in the intestinal tract as well as in the lymphoid tissues.

The pattern of the lesion found in the lymphoid tissues in this experiment was similar to that in the preliminary study i.e. initial lymphocytolysis (present at 5 DPI) with subsequent depletion and, in the nodes, GALT and spleen, regeneration (commencing by 6-7 DPI and advanced by 12-13 DPI).

The impression gained from the preliminary study that thymic degeneration and atrophy was an important and quantifiable change was borne out by the results of this experiment. Destruction and depletion of the thymus was again a consistent macroscopic and microscopic feature. The loss of thymic mass was even more striking when expressed in terms of thymic/body weight ratios, with marked differences from control animals present even at 5 DPI.

In view of the extensive lymphoid destruction and depletion present at 5, 6 and 7 DPI, it is rather surprising that circulating antibody first appears and rises rapidly at this time.

Although vomiting had been a major clinical sign, there was no evidence of an active gastritis. The main finding in the stomach of infected dogs was the presence of a large volume of mucoid fluid. The source of the fluid is unclear. Since the dogs were not only anorexic but also refused fluids, the gastric fluid must have been derived from body secretions. The fluid was occasionally bile-stained, suggesting some reflux from the duodenum, but in the majority of cases it was clear, indicating either a gastric or salivary origin or a mixture of the two. The accumulation of the fluid probably reflects a failure of normal gastric emptying through the pylorus.

The striking difference in the pathological findings between this experiment and the preliminary study was the presence of intestinal lesions in all animals.

The main lesions were present in the small intestine. Thickening and increased rigidity of the intestinal coils were the most striking macroscopic features. It was interesting that serosal granularity was most marked in the dogs which had died, indicating that this finding, so noticeable in field cases, is a terminal feature. Similarly, the red/purple mottling of the spleen was only noted in dogs which died.

Microscopical changes in the small intestine could be divided into a number of easily recognised patterns. The varying occurrence of these patterns at different times after infection illuminates the sequential development of the characteristic lesions seen in field outbreaks. Pattern A, in which least disruption of intestinal architecture was apparent, would appear to be an early change. This pattern has not been recognised in field cases and was only observed in this

experiment at 5 DPI. The other patterns were identical to those found in field cases but this experiment allows them to be placed sequentially.

Pattern B, with stunting of villi and crypt dilation or epithelial attenuation, was the major pattern at 5 and 6 DPI, while pattern C, with its extensive disruption of mucosal architecture, was seen only at 7 DPI and only in the dogs which were found dead. Early evidence of regeneration was present in the dog killed at 7 DPI and by 12 and 13 DPI the regenerative pattern D was exclusively present.

The basis of the intestinal lesions would appear to be destruction of cells in the proliferative zone of the intestinal crypts with shortening of villi, attenuation of epithelium and the later hyperplastic crypt reaction being methods of maintaining and restoring intestinal integrity. The mucosal destruction of pattern C, found in dogs which died, would appear to be the end result of failure of these mechanisms.

While the development of lesions in the small intestine as a whole appeared to have followed the same course, i.e. A to B to C or D, it was apparent that within individual animals, different patterns of lesion were found at different levels of the small intestine. On examination of the data, it was clear that this variation was not random but that changes at any given time in the upper small intestine (duodenum and jejunum) were more advanced than those in the ileum. Further, at 5 and 6 DPI, the extent of intestinal lesions also varied in this way, with lesions in the duodenum and jejunum being widespread or even confluent while changes in the ileum were focal except over the GALT.

A possible explanation for this spatial variation in lesion pattern lies in the fact that there may be a smaller pool of proliferating cells in the distal small intestine than in the upper. This is true in rodents (Clarke, 1970, 1971) and in cats

(McMinn 1954; Carlson et al., 1977b) and seems likely to be the case in other mammalian species including the dog. Since CPV has a known propensity for actively dividing cells, it would be expected that damage would appear earlier and be more extensive in the more mitotically active upper areas of the small intestine. A relatively low mitotic rate in the caecum and colon would also be the most reasonable explanation for the limited lesions present in those areas.

Estimation of the severity of epithelial lesions in areas immediately overlying the GALT was difficult. There was epithelial attenuation and dilation of crypts, indicating that epithelial damage had occurred; but indirect effects, such as local tissue collapse secondary to lymphoid destruction, may also have contributed to distortion of these areas. However, Carlson and his co-workers (1977a and b) observed more extensive lesions in the mucosa adjacent to the GALT in the ileum of cats infected with FPV. They postulated that this could be a result of increased virus/cell contact directly due to the high concentration of virus present in the lymphoid tissue.

Although this study allowed a detailed examination of the lesions associated with severe clinical disease, certain questions remain unanswered. Chief among these is how CPV reaches the intestinal epithelia and at what stage of infection it does so. Certainly, from this study, virus was present in intestinal epithelia by 5 DPI but whether the route was direct from the intestinal lumen or indirect following systemic spread and lymphoid replication is unclear. The latter would seem more likely in view of the advanced lymphoid lesions present even at 5 DPI in this experiment and at 3 DPI in the preliminary study. Resolution of these questions still requires detailed investigation (including immunocytochemistry) in the first few days after infection.

The outstanding success of this experiment was the reproduction of severe disease in the experimental animals. The factors which may have contributed to this are threefold. First, the infecting virus had not been passaged through tissue culture. Second, a very large dose of virus was used. Third, the dogs were younger, eight weeks of age as opposed to 12 weeks in the preliminary study.

The first two factors may have improved the quality of challenge, as was, indeed, intended and as has already been discussed in Chapter 4 above.

The importance of age in relation to severity of disease following experimental challenge has not been considered by other workers although, in field outbreaks of disease, the mortality is significantly higher in pups under 12 weeks of age than in older animals. Dramatic alterations in normal mammalian intestinal cell kinetics occur in the period of weaning (Koldovsky et al., 1966) when, in association with the change in diet, feeding regimes and intestinal flora, there is an increase in the mitotic activity of intestinal epithelia, which gradually falls following weaning to a lower and steadier adult level. In view of the inherent proclivity of parvoviruses to replicate in dividing cells, it would be expected that infection at the time of the post-weaning mitotic surge would be more likely to result in severe enteric disease than infection at a later stage.

The explanation of the dramatic differences in severity of enteric disease between the dogs in this experiment and in the preliminary study may therefore be that although the pups were obtained from the same source (which weans at six weeks of age), the pups in the preliminary study were infected at 12 weeks of age i.e. six weeks post-weaning, while in this investigation, they were infected at eight weeks of age i.e. two weeks post-weaning when a higher intestinal mitotic index might have been expected.

TABLE 18.

CHAPTER 6 : EXPERIMENTAL DESIGN.

	Dog Number	Day Killed	
Group 1	11	0	Control
	12	0	
Group 2	13	5	Infected
	14	5	
	15	6	
	16	6	
	17	6	
	18	6 died	
	19	6	
	20	7 died	
	21	7	
	22	7 died	
	23	12	
	24	13	

TABLE 19.

CHAPTER 6: CLINICAL SIGNS

Dog No.	DPT													
	0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	-													
12	-													
13	-	-	-	-	A++	A+++V								
						D+++								
14	-	-	-	-	A++	A+++V								
						D+++								
15	-	-	-	-	-	A++	A++							
							D++							
16	-	-	-	-	-	A+	A++V							
							D+++							
17	-	-	-	-	A+	A+	A++							
							D++							
18	-	-	-	-	-	A+++V	Died							
						D+								
19	-	-	-	-	-	A+	A+++							
							D++							

TABLE 19 (CONTINUED)

CHAPTER 6: CLINICAL SIGNS

Dog No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13
							DPT							
20	-	-	-	-	-	A+	A++V	Died						
							D++							
21	-	-	-	-	-	A+	A+	A+V						
							D+	D++						
22	-	-	-	-	-	A++	A++	Died						
							D++							
23	-	-	-	-	-	A+V	A++V	-	-	-	-	-	-	-
							D+							
24	-	-	-	-	-	A+V	A++V	-	-	-	-	-	-	-
							D+							

A = Dullness and Anorexia. D = Diarrhoea. + = Mucoïd. ++ = Fluid. +++ = Dysenteric. V = Vomition.
+ to +++ on severity.

TABLE 20.

CHAPTER 6 : TOTAL CIRCULATING LEUKOCYTE COUNTS ($\times 10^9/\text{Litre}$).

Dog	Day Post Infection														
No.	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	13.9	12.9													
12	15.4	19.7													
13	19.5	17.7	ND	ND	ND	ND	7.8								
14	16.4	15.6	ND	ND	ND	ND	10.5								
15	14.7	16.2	ND	ND	ND	ND	12.6	2.9							
16	15.8	17.9	ND	ND	ND	ND	10.4	2.5							
17	ND	16.7	ND	ND	ND	ND	7.0	2.7							
18	17.6	ND	ND	ND	ND	ND	8.1	ND							
19	13.5	11.4	ND	ND	ND	ND	ND	1.1							
20	14.9	13.1	ND	ND	ND	ND	14.8	ND	ND						
21	14.8	18.4	ND	ND	ND	ND	19.3	ND	10.2						
22	ND	17.9	ND	ND	ND	ND	ND	ND	ND						
23	15.7	18.0	ND	ND	ND	ND	11.0	ND	ND	ND	24.2	ND	ND	15.2	
24	ND	18.8	ND	ND	ND	ND	17.6	ND	ND	ND	32.8	ND	ND	ND	28.8

ND = Not Done.

TABLE 21.

CHAPTER 6 : ABSOLUTE CIRCULATING NEUTROPHIL COUNTS ($\times 10^9/\text{Litre}$)

Dog No.	Day Post Infection														
	-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	10.2	9.5													
12	11.6	14.0													
13	13.6	12.1	ND	ND	ND	ND	4.36								
14	12.3	11.6	ND	ND	ND	ND	9.13								
15	11.2	10.0	ND	ND	ND	ND	9.95	0.72							
16	9.0	9.8	ND	ND	ND	ND	8.32	0.842							
17	ND	10.5	ND	ND	ND	ND	5.04	0.864							
18	13.7	ND	ND	ND	ND	ND	5.26	ND							
19	9.6	7.4	ND	ND	ND	ND	8.5	0.365							
20	9.7	8.0	ND	ND	ND	ND	12.6	ND	ND						
21	9.3	11.0	ND	ND	ND	ND	15.0	ND	2.85						
22	ND	11.4	ND	ND	ND	ND	ND	ND	ND						
23	12.1	13.7	ND	ND	ND	ND	7.81	ND	ND	ND	11.85	ND	ND	9.88	
24	11.0	14.7	ND	ND	ND	ND	15.6	ND	ND	ND	20.0	ND	ND	ND	21.6

ND = Not Done

TABLE 22.

CHAPTER 6 : ABSOLUTE CIRCULATING LYMPHOCYTE COUNTS ($\times 10^9/\text{Litre}$).

Dog No.	- 1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	3.6	3.3													
12	3.8	5.2													
13	5.9	5.4	ND	ND	ND	ND	3.35								
14	4.1	3.7	ND	ND	ND	ND	0.525								
15	3.5	5.9	ND	ND	ND	ND	1.9	1.13							
16	6.7	7.9	ND	ND	ND	ND	1.66	1.3							
17	ND	6.0	ND	ND	ND	ND	1.68	1.24							
18	3.9	ND	ND	ND	ND	ND	2.5	ND							
19	3.9	3.9	ND	ND	ND	ND	0.5	0.6							
20	5.2	5.0	ND	ND	ND	ND	1.8	ND	ND						
21	5.5	7.4	ND	ND	ND	ND	3.66	ND	6.01						
22	ND	6.3	ND	ND	ND	ND	ND	ND	ND						
23	3.5	4.0	ND	ND	ND	ND	2.31	ND	ND	ND	11.61	ND	ND	3.95	
24	ND	3.9	ND	ND	ND	ND	1.6	ND	ND	ND	12.13	ND	ND	ND	6.04

ND = Not Done.

TABLE 23.

CHAPTER 6: EXAMINATION OF FAECAL SPECIMENS AND INTESTINAL
CONTENTS FOR THE PRESENCE OF SPECIFIC CPV HAEMAGGLUTININ*

Dog		Day Post Infection												
No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	< 2													
12	< 2													
13	< 2	ND	ND	ND	ND	1024 (C = 64)								
14	< 2	ND	ND	ND	ND	512 (C = 128)								
15	< 2	ND	ND	ND	ND	128		2 (C=<2)						
16	< 2	ND	ND	ND	ND	2048		8 (C=<2)						
17	< 2	ND	ND	ND	ND	ND-		16 (C=<2)						
18	< 2	ND	ND	ND	ND	ND		8 (C=<2)						
19	< 2	ND	ND	ND	ND	256		1024 (C=ND)						
20	< 2	ND	ND	ND	ND	64		256		512 (C=2)				
21	< 2	ND	ND	ND	ND	ND		256		< 2 (C=<2)				
22	< 2	ND	ND	ND	ND	ND		ND		1024 (C=<2)				
23	< 2	ND	ND	ND	ND	256		ND		4	4	ND	ND	< 2 (C=<2)
24	< 2	ND	ND	ND	ND	ND		4		ND	ND	ND	< 2	2 (C=<2)

C = Intestinal contents. ND = Not Done

* Results expressed as the reciprocal of the highest dilution giving complete agglutination.

TABLE 24.

CHAPTER 6: EXAMINATION OF FAECAL SWABS FOR THE PRESENCE OF SPECIFIC CPV HAEMAGGLUTININ*

Dog No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	<2													
12	<2													
13	<2	ND	ND	ND	ND	512								
14	<2	ND	ND	ND	ND	32								
15	<2	ND	ND	ND	ND	512	8							
16	<2	ND	ND	ND	ND	64	16							
17	<2	ND	ND	ND	ND	64	4							
18	<2	ND	ND	ND	ND	ND	8							
19	<2	ND	ND	ND	ND	16	8							
20	<2	ND	ND	ND	ND	32	128	64						
21	<2	ND	ND	ND	ND	1024	512	<2						
22	<2	ND	ND	ND	ND	2048	256	1024						
23	<2	ND	ND	ND	ND	4	32	ND	<2	4	ND	ND	<2	
24	<2	ND	ND	ND	ND	2	8	ND	ND	<2	ND	ND	ND	<2

* Results are expressed as reciprocal of the highest dilution giving complete agglutination.

ND = Not Done.

TABLE 25.

CHAPTER 6 : EXAMINATION OF SERA FOR THE PRESENCE OF HAEMAGGLUTINATION INHIBITING ANTIBODY TO CPV*

Dog No.	0	Day Post Infection												
		1	2	3	4	5	6	7	8	9	10	11	12	13
11	< 4													
12	< 4													
13	< 4	ND	ND	ND	ND	< 4								
14	< 4	ND	ND	ND	ND	128								
15	< 4	ND	ND	ND	ND	64	512							
16	< 4	ND	ND	ND	ND	< 4	1024							
17	< 4	ND	ND	ND	ND	256	1024							
18	< 4	ND	ND	ND	ND	128	ND							
19	< 4	ND	ND	ND	ND	8	128							
20	< 4	ND	ND	ND	ND	< 4	16	ND						
21	< 4	ND	ND	ND	ND	16	512	2048						
22	< 4	ND	ND	ND	ND	< 4	64	ND						
23	< 4	ND	ND	ND	ND	256	ND	ND	ND	1024	ND	ND	2048	
24	< 4	ND	ND	ND	ND	16	ND	ND	ND	1024	ND	ND	ND	1024

* Results are expressed as the reciprocal of the highest dilution which completely inhibited haemagglutination.

TABLE 26.

CHAPTER 6 : EXAMINATION OF INTESTINAL CONTENTS FOR THE PRESENCE OF HAEMAGGLUTINATION INHIBITING ANTIBODY TO CPV*

Dog No.	0	1	2	3	4	5	6	7	8	9	10	11	12	13
11	< 4													
12	< 4													
13	-	-	-	-	-	< 4								
14	-	-	-	-	-	< 4								
15	-	-	-	-	-	-	64							
16	-	-	-	-	-	-	64							
17	-	-	-	-	-	-	16							
18	-	-	-	-	-	-	8							
19	-	-	-	-	-	-	ND							
20	-	-	-	-	-	-	-	16						
21	-	-	-	-	-	-	-	64						
22	-	-	-	-	-	-	-	4						
23	-	-	-	-	-	-	-	-	-	-	-	-	< 4	
24	-	-	-	-	-	-	-	-	-	-	-	-	-	< 4

* Results are expressed as the reciprocal of the highest dilution which completely inhibited haemagglutination.

TABLE 27.

CHAPTER 6: POST MORTEM FINDINGS

Dog No.	Day Killed	Macroscopic Findings
11	0	NAD
12	0	NAD
13	5	Small thymus, oedematous lymph nodes, intestinal wall thickened, congested. Duodenum most severely affected.
14	5	Small thymus, oedematous lymph nodes, intestine wall thickened, congested. Duodenum most severely affected.
15	6	Small thymus, oedematous lymph nodes, intestinal wall thickened, congested. Duodenum most severely affected.
16	6	Small thymus, oedematous lymph nodes. Intestinal wall thickened, congested. Jejunum most severely affected.
17	6	Small thymus, oedematous lymph nodes, intestinal wall thickened, congested. Jejunum most severely affected.
18	6 Died	Small thymus, oedematous lymph nodes, intestinal wall thickened, congested, salt-glaze serosa, jejunum most severely affected. Mottled spleen.

TABLE 27 (CONTINUED)

CHAPTER 6: POST MORTEM FINDINGS

Dog No.	Day Killed	Macroscopic Findings
19	6	Small thymus, oedematous lymph nodes, intestinal wall thickened, congested, jejunum most severely affected.
20	7 Died	Small thymus, oedematous lymph nodes, intestinal wall thickened, congested. Salt glaze serosa, duodenum most severely affected. Mottled spleen.
21	7	Small thymus, oedematous lymph nodes, intestinal wall thickened, congested. Duodenum most severely affected
22	7 Died	As Dog 20
23	12	Intestinal wall slightly thickened, lacy thymus, enlarged lymph nodes.
24	13	Intestinal wall slightly thickened, lacy thymus, enlarged lymph nodes.

TABLE 28.

CHAPTER 6 : QUANTIFICATION OF TOTAL BODY WEIGHT AND THYMIC WEIGHT, TOGETHER WITH THE BODY WEIGHT TO THYMIC WEIGHT RATIO

Dog No.	DPI Killed	Body Wt. (g)	Thymic Wt. (g)	<u>Body Wt.</u> Thymus Wt.
11	0	2060	8	257.5
12	0	2035	8.5	239.4
13	5	1818	5	363.6
14	5	3181	7	454.4
15	6	3409	6	568.1
16	6	3181	6.5	489.4
17	6	2440	6	406
18	6 died	2668	5.5	485
19	6	2897	7	413.8
20	7 died	2954	6.5	454.4
21	7	3238	5	647.6
22	7 died	2895	6	482.5
23	12	4232	6	705
24	13	2240	1g	2240

TABLE 29.

CHAPTER 6: CLASSIFICATION OF INTESTINAL LESIONS

Dog No.	Day Killed	Duodenum		Jejunum		Ileum	
		Pattern	Confluency	Pattern	Confluency	Pattern	Confluency
13	5	R	+++	B	++	A	+
14	5	B	++	A/B	++	A	+
15	6	R	+++	B	+++	B	+
16	6	B	++	B	+++	B	+
17	6	R	++	B	+++	B	+
18	6 Died	B	++	B	+++	B	++
19	6	B	++	B	+++	B	++
20	7 Died	C	+++	C	+++	B	+++
21	7	B/D	+++	B	++	B	++
22	7 Died	C	+++	C	+++	R	+++
23	12	D	++	D	++	D	+
24	13	D	++	D	++	D	+

Fig. 29: Canine Parvovirus Infection - Dogs 13, 15, 16 and 19.

Note the marked depression of Dog 13 (arrowed), when compared to the demeanor of dogs 15, 16 and 19. Dogs were placed in contact for photography. Taken at 4 DPI.

Fig. 30: Canine Parvovirus Infection - Dogs 14 and 24.

Dog 14 (arrowed) is dull and dehydration, evinced by persistent tenting of dorsal skin, is a prominent clinical sign. Dog 24 is slightly dull, although dehydration is not a feature. Taken at 5 DPI.



Fig. 31: Canine Parvovirus Infection - Dog 15.

The animal is prostrate, and the eyes sunken. Fluid blood-stained faeces are passed, without apparent effort. Taken on the morning of 6 DPI.



Fig. 32: Canine Parvovirus Infection - Blood Smear of Dog 15.

Note the granular nucleus and deeply basophilic cytoplasm of this activated lymphocyte. Relatively large numbers of this cell type were present in the peripheral blood. Taken at 6 DPI.

May-Grunwald-Giemsa Stain
x 1,000.

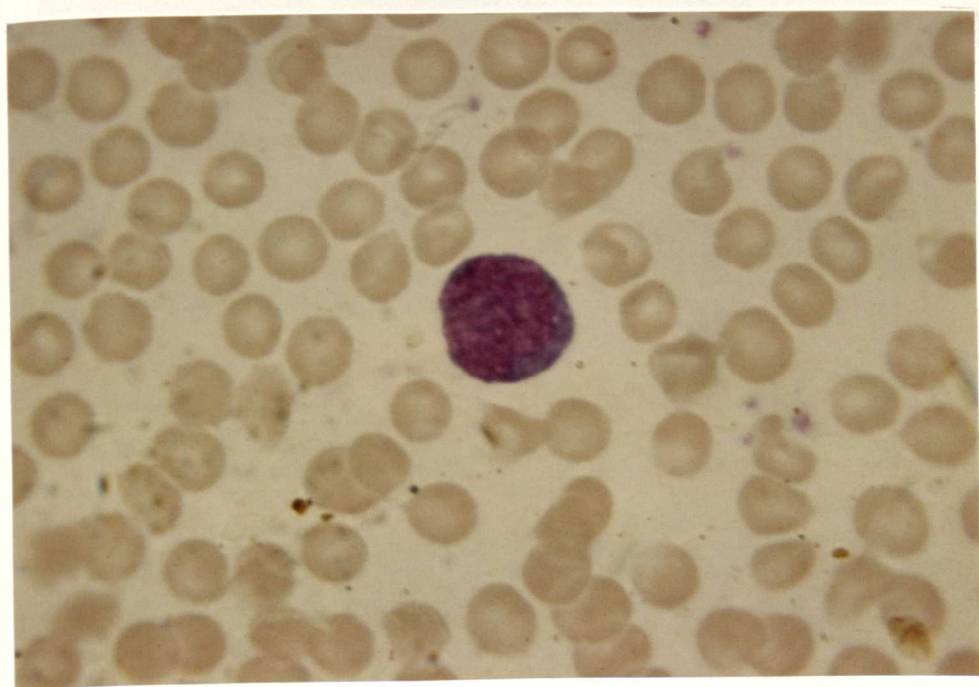


Fig. 33: Canine Parvovirus Infection - Duodenum of Dog 15.

The wall of the duodenum is thickened, and appears oedematous. The contents are scant and mucoid. Taken at 6 DPI.

Fig. 34: Canine Parvovirus Infection - Stomach of Dog 15.

The stomach contains large amounts of thick, clear mucus. The mucosa itself appears normal. Taken at 6 DPI.

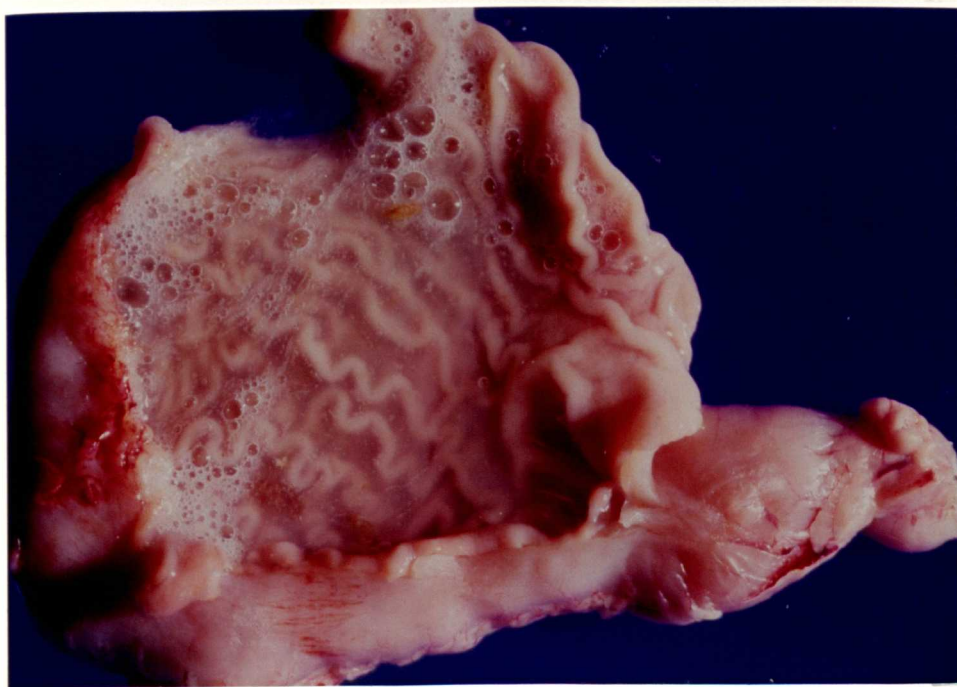
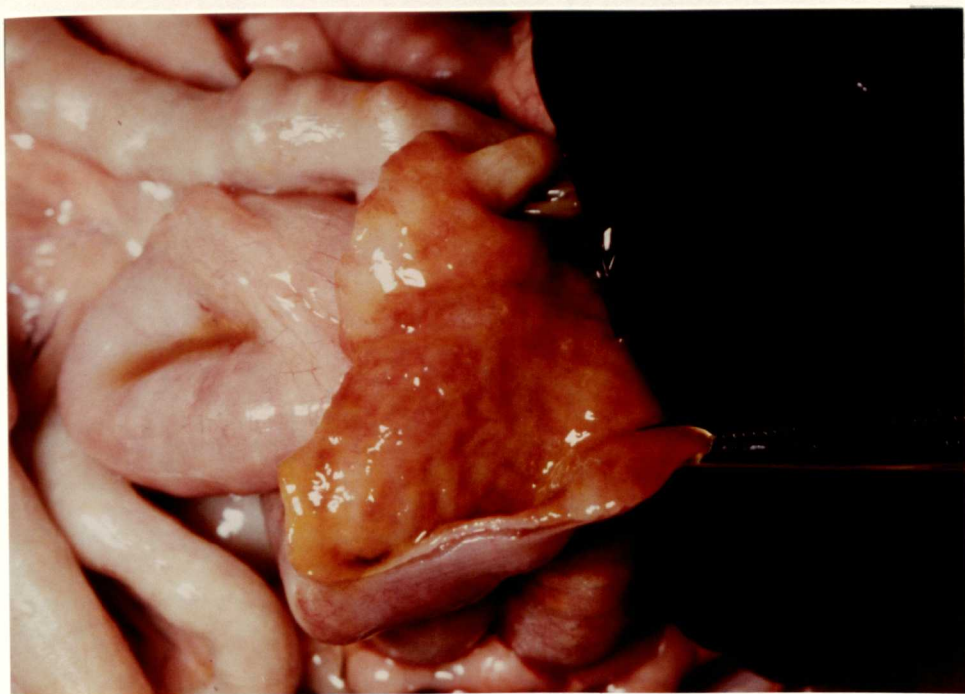


Fig. 35: Canine Parvovirus Infection - Intestine of Dog 14.

The mucosa over the GALT appears necrosed, and is congested. Taken at 5 DPI.

Fig. 36: Canine Parvovirus Infection - Thymus of Dog 13

The thymus is slightly smaller than normal, and is somewhat gelatinous in appearance. Taken at 5 DPI.

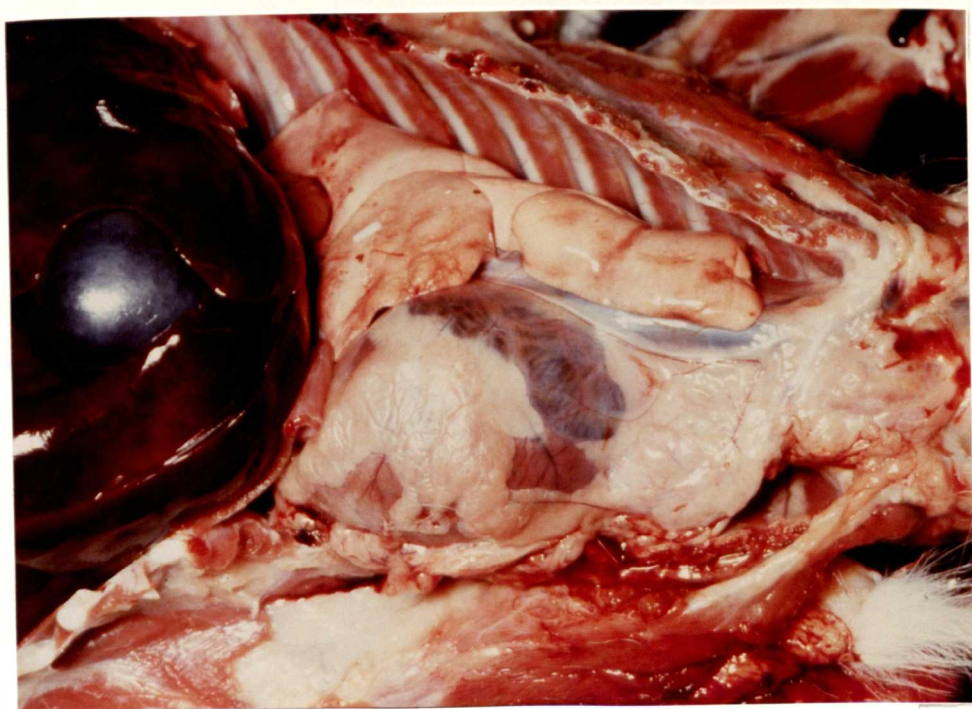


Fig. 37: Thymus of Dog 11.

The lobules of the thymus are separated by a delicate connective tissue stroma, and are clearly divisible into a cortex and medulla. Control dog.

H & E x 40.

Fig. 38: Canine Parvovirus Infection - Thymus of Dog 21.

The cortex is depleted, and consequently stains less densely than the medulla, a reversal of the normal pattern. Taken at 7 DPI.

H & E x 40.

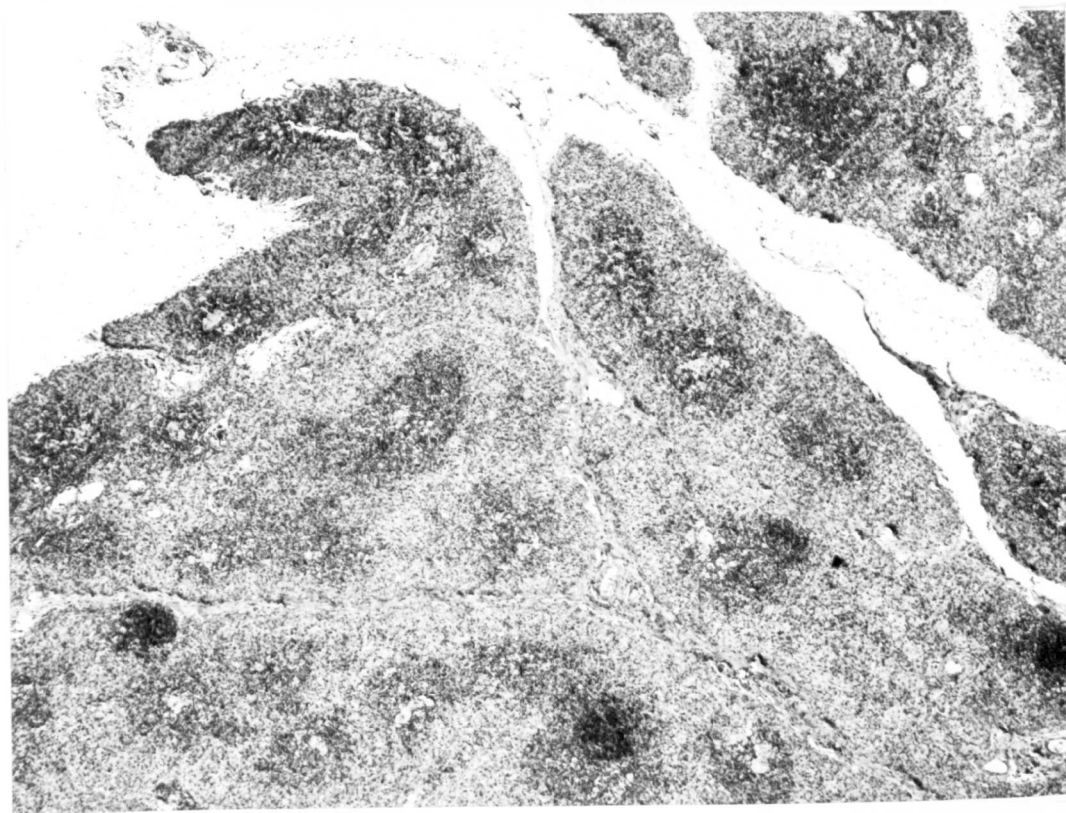
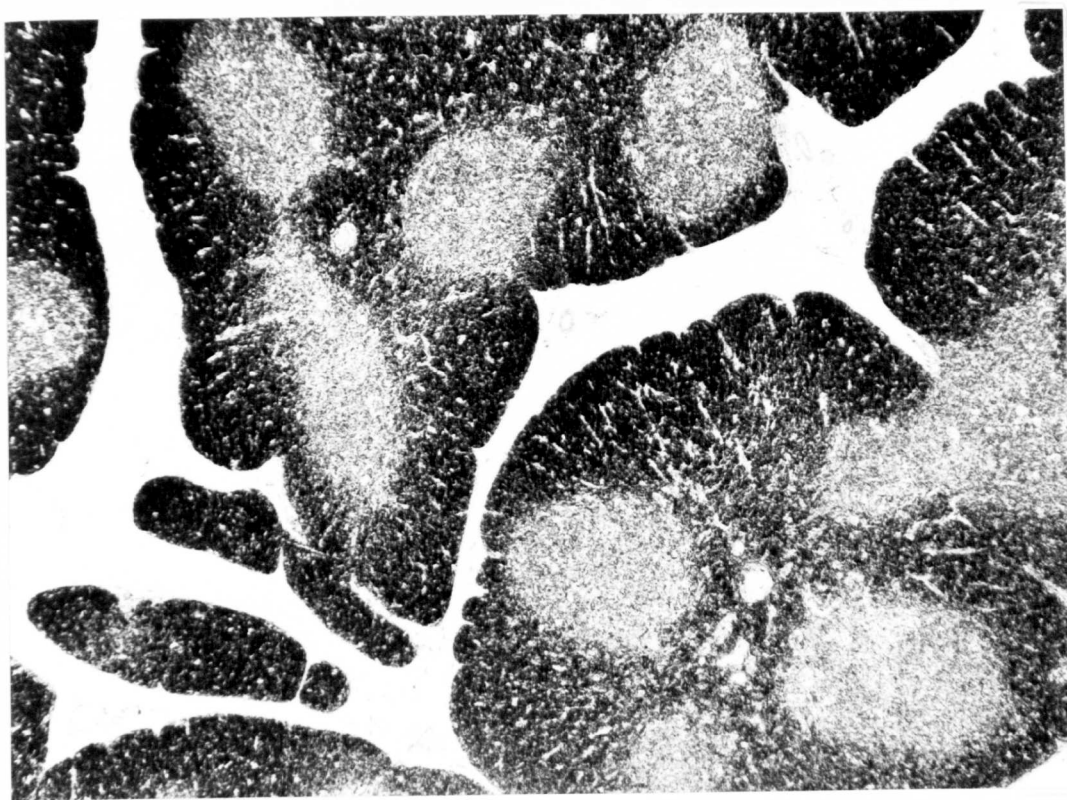


Fig. 39: Canine Parvovirus Infection - Thymus of Dog 24.

The cortex is depleted although it contains foci of lymphoid cells. The medulla is hypercellular. Taken at 13 DPI.

H & E x 40.

Fig. 40: Canine Parvovirus Infection - Lymph Node of Dog 11.

In this normal lymph node there are numerous germinal centres in the cortex. The paracortex is relatively narrow and the medullary cords are well defined.

H & E x 40.

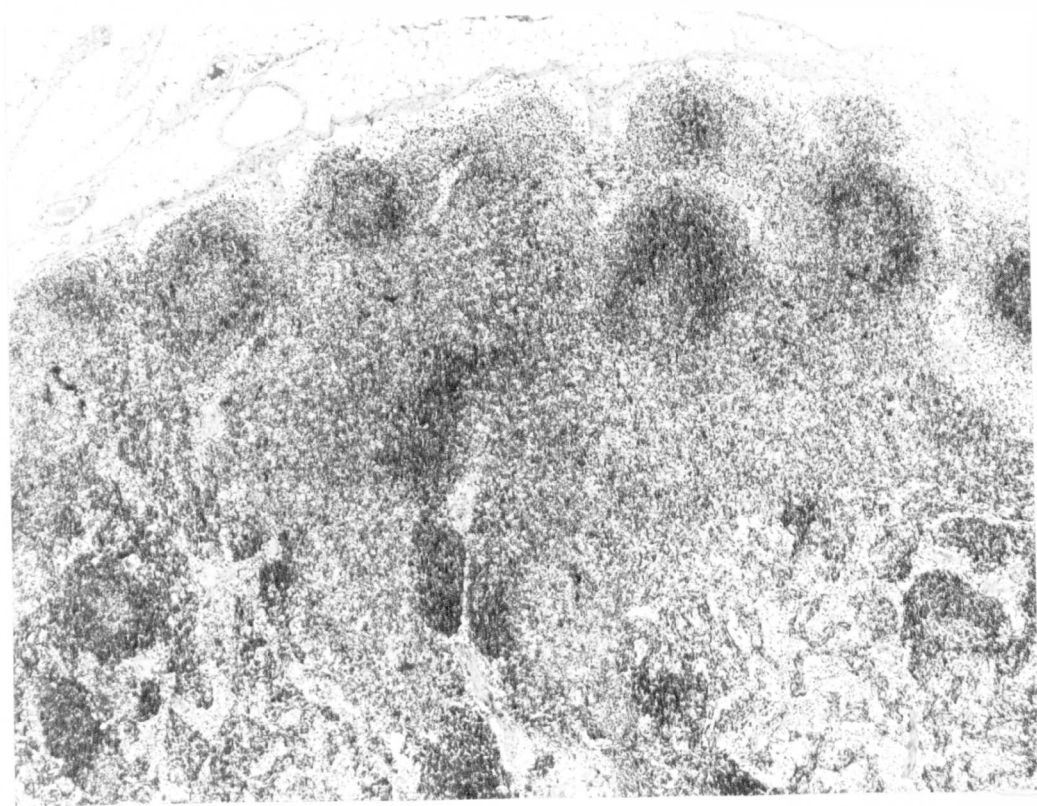
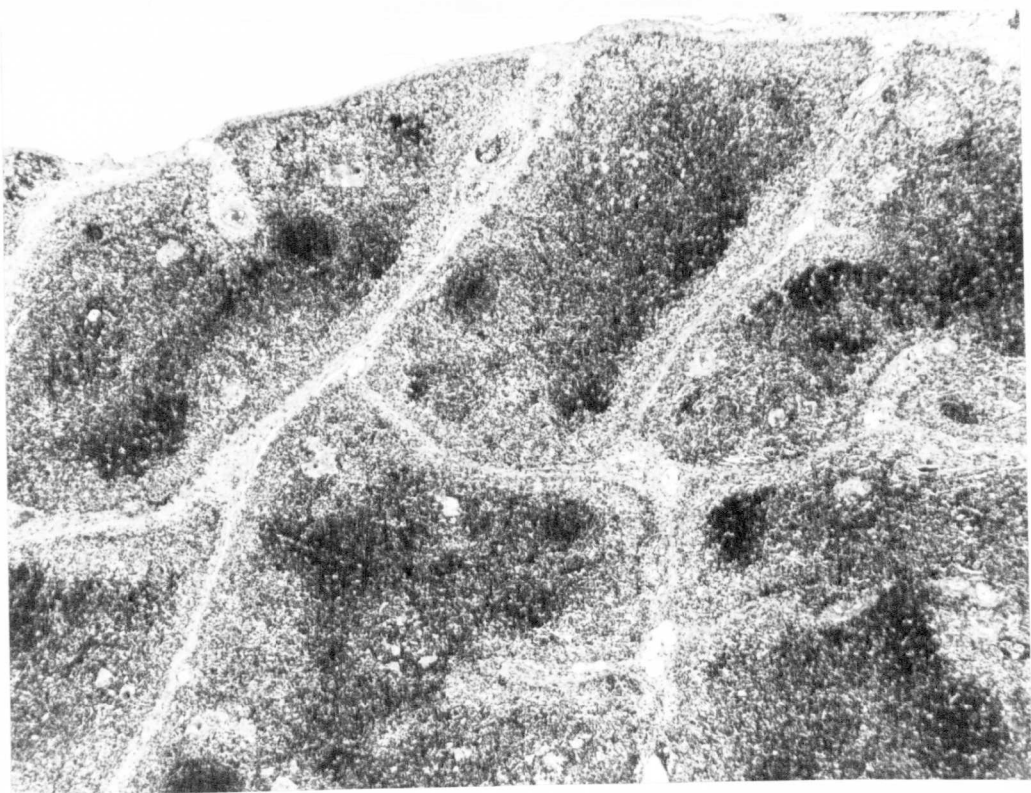


Fig. 41: Canine Parvovirus Infection - Lymph Node of Dog 14.

The germinal centre is hypocellular and is composed basically of histiocytic reticulum cells. However, there is still pyknosis of cells centrally.

Taken at 5 DPI.

H & E x 250.

Fig. 42: Canine Parvovirus Infection - Lymph Node of Dog 13

The germinal centre is depleted being composed of histiocytic reticulum cells. Some neutrophils were present in the centre. Taken at 5 DPI.

H & E x 250.

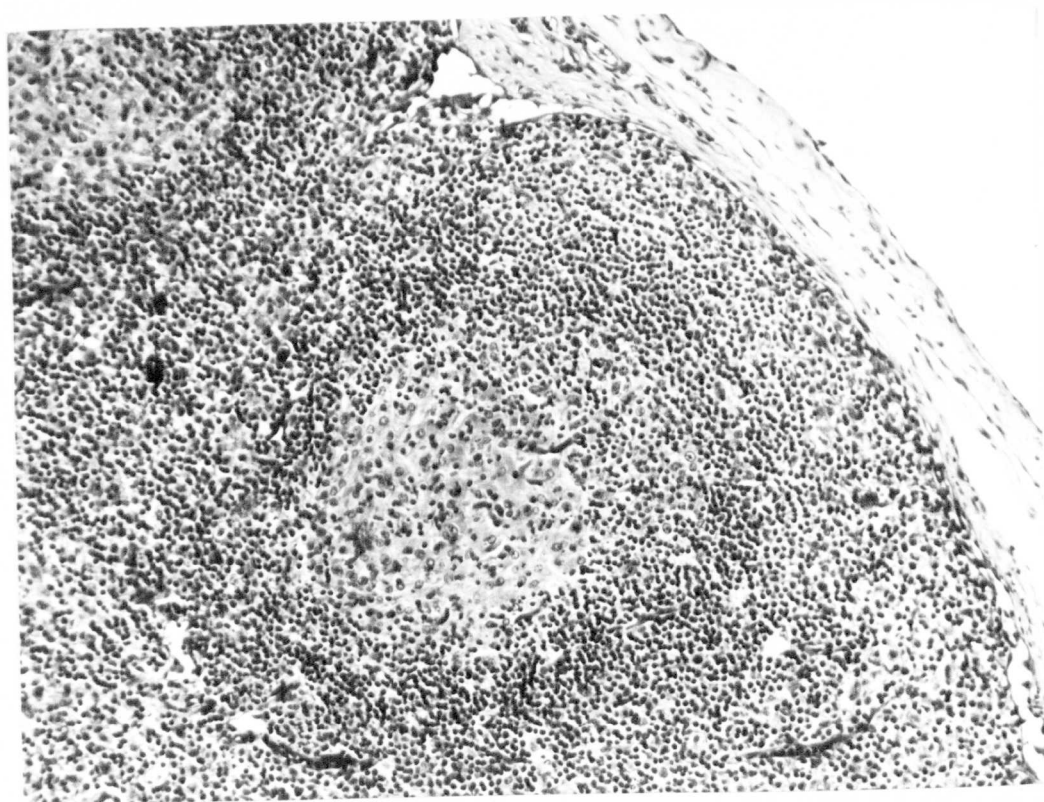
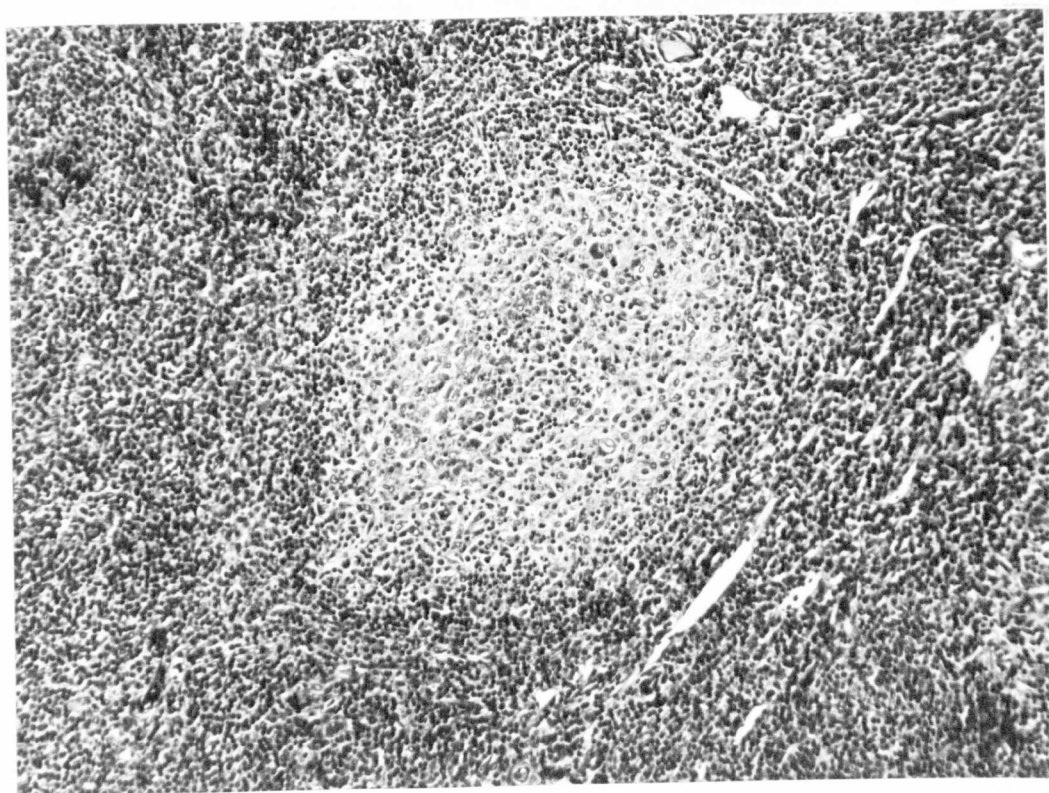
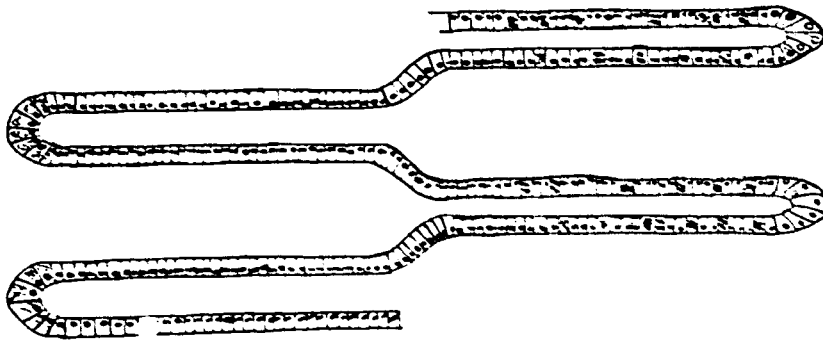
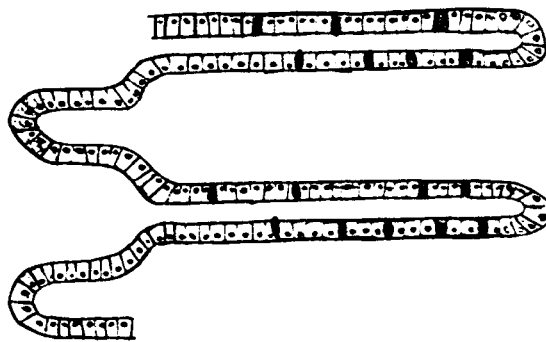


Fig. 43:

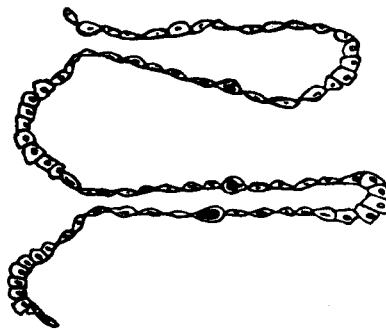
Canine Parvovirus Infection - Patterns of Intestinal Lesions.



Normal



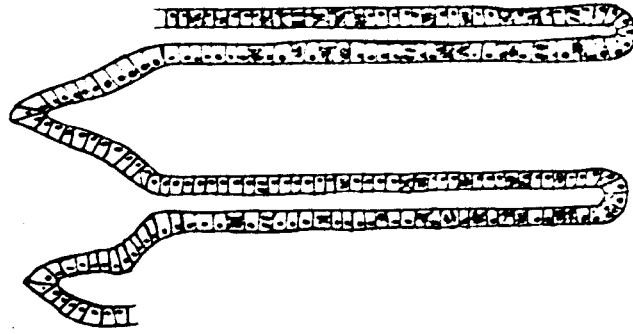
Pattern A



Pattern B



Pattern C



Pattern D

Fig. 44: Canine Parvovirus Infection - Jejunum of Dog 11.

In this normal jejunum the villi are tall and slender with a central lacteal. The epithelium lining the crypts is intensely basophilic.

H & E x 100.

Fig. 45: Canine Parvovirus Infection - Duodenum of Dog 13.

The crypts are lined by an attenuated epithelium in which large amphophilic intranuclear inclusion bodies are apparent. The basal cells are unaffected. Taken at 5 DPI.

H & E x 250.

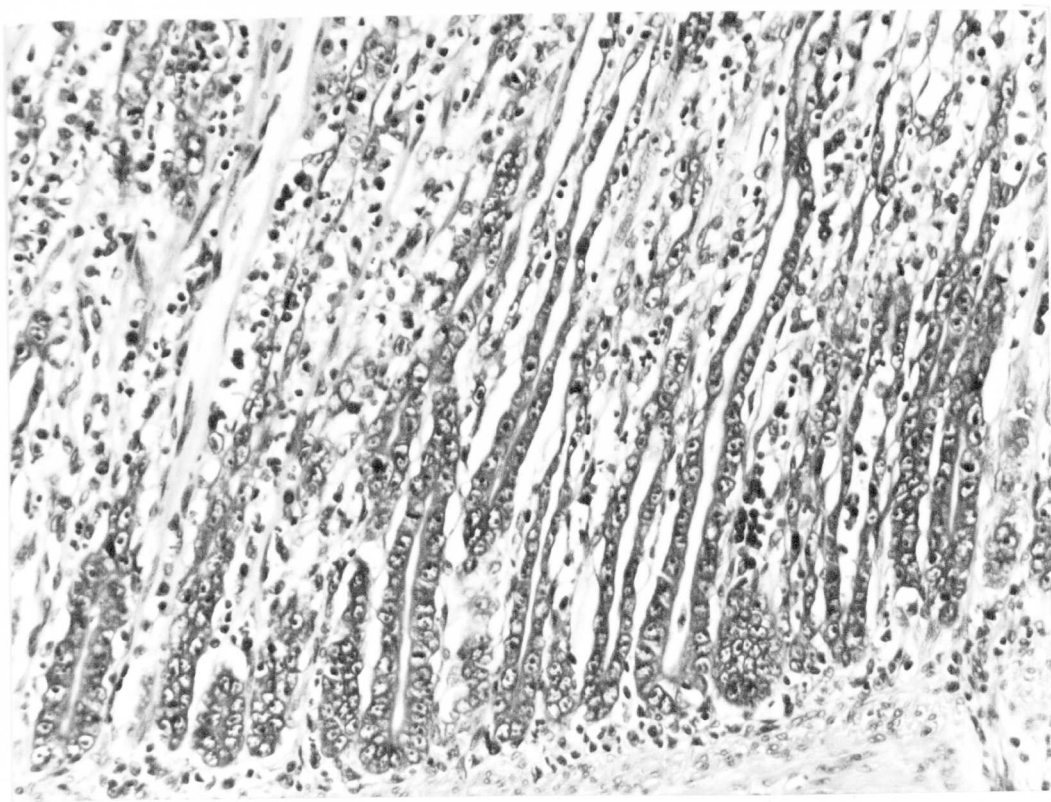
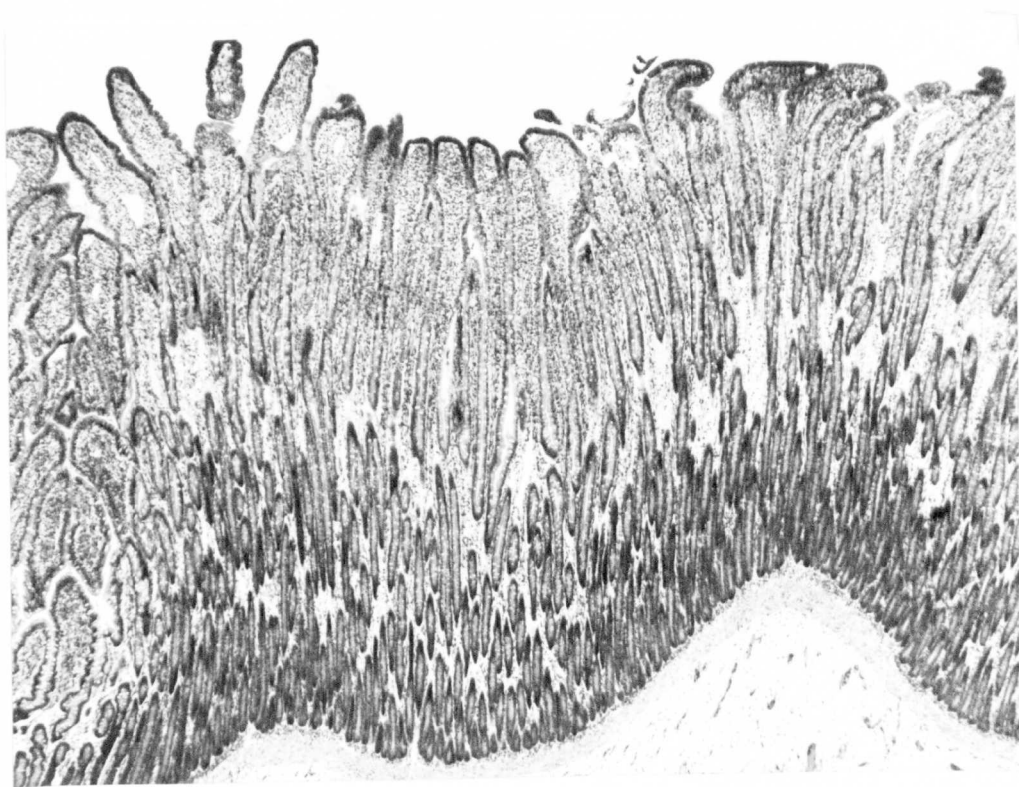


Fig. 46: Canine Parvovirus Infection - Duodenum of Dog 15.

The villi are greatly stunted, although mostly still covered by an intact epithelium. The crypts are lined by an attenuated epithelium, and the lumina are dilated. Taken at 6 DPI.

H & E x 100.

Fig. 47: Canine Parvovirus Infection - Duodenum of Dog 20.

There is complete loss of villi although some are represented by domed protuberances. The crypt architecture is obliterated by collapse of the mucosa, however some dilated crypt remnants may be discerned. Taken at 7 DPI.

H & E x 100.

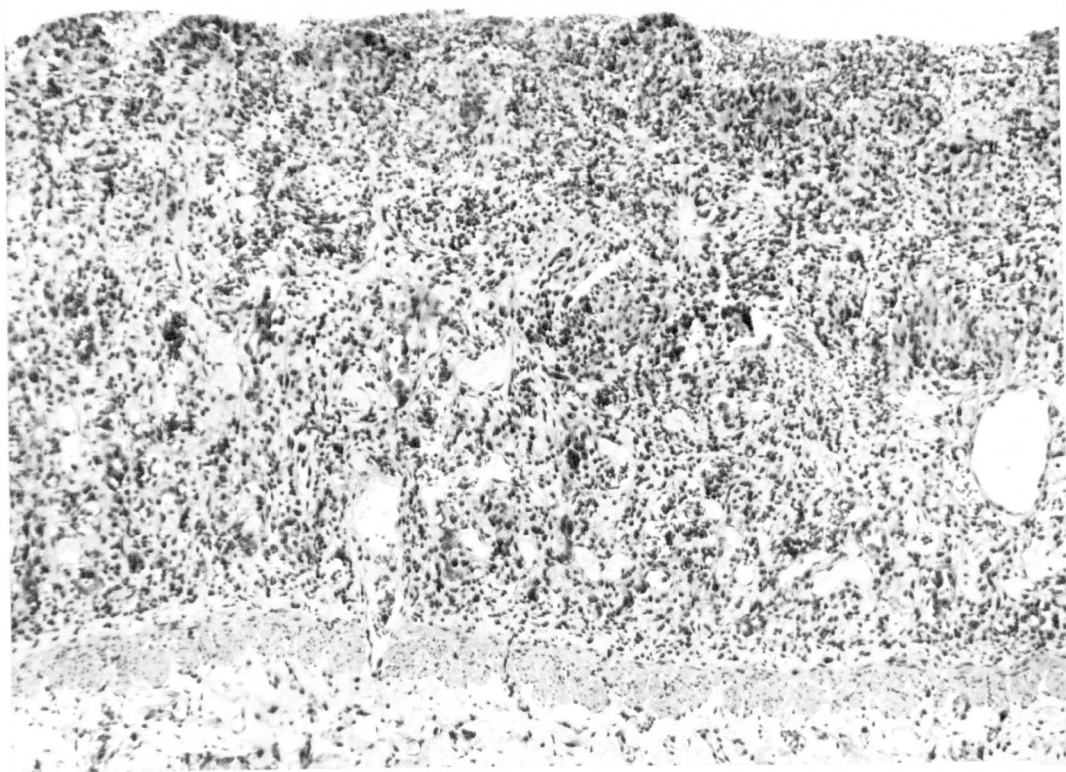
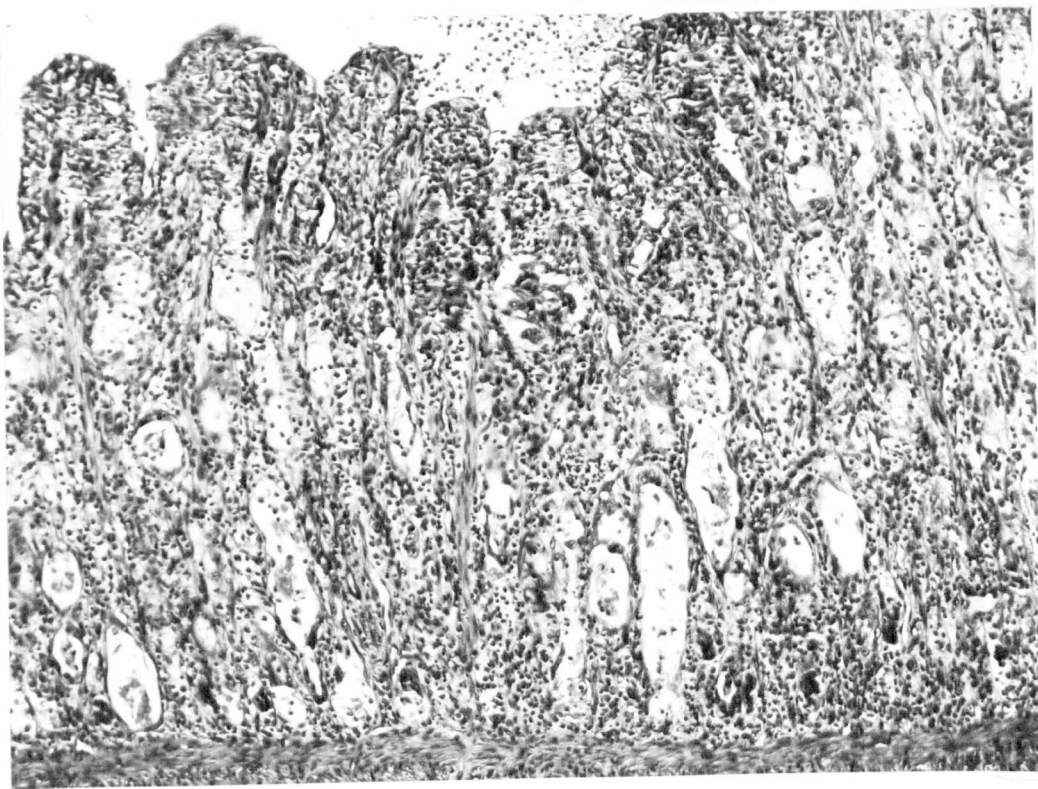


Fig. 48: Canine Parvovirus Infection - Jejunum of Dog 24.

The crypts are lined by a basophilic epithelium. The villi are short and appear conical. Numerous goblet cells were present in the epithelium of the crypts and villi.

H & E x 40.

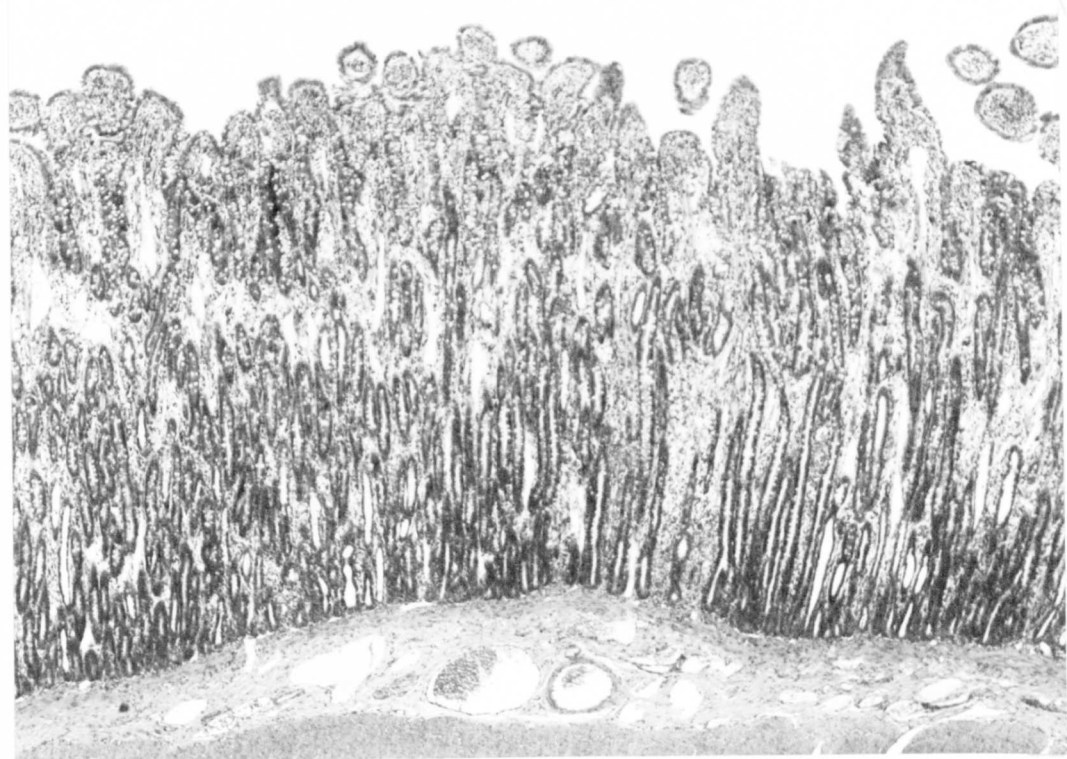


Fig. 49: Canine Parvovirus Infection - Duodenum of Dog 21.

Some crypts are dilated and lined by an attenuated epithelium. However, in others, the epithelium is cuboidal and darkly basophilic, suggesting an attempt at epithelial regeneration. Taken at 7 DPI.

H & E x 40.



Fig. 50: Marrow of Dog 11.

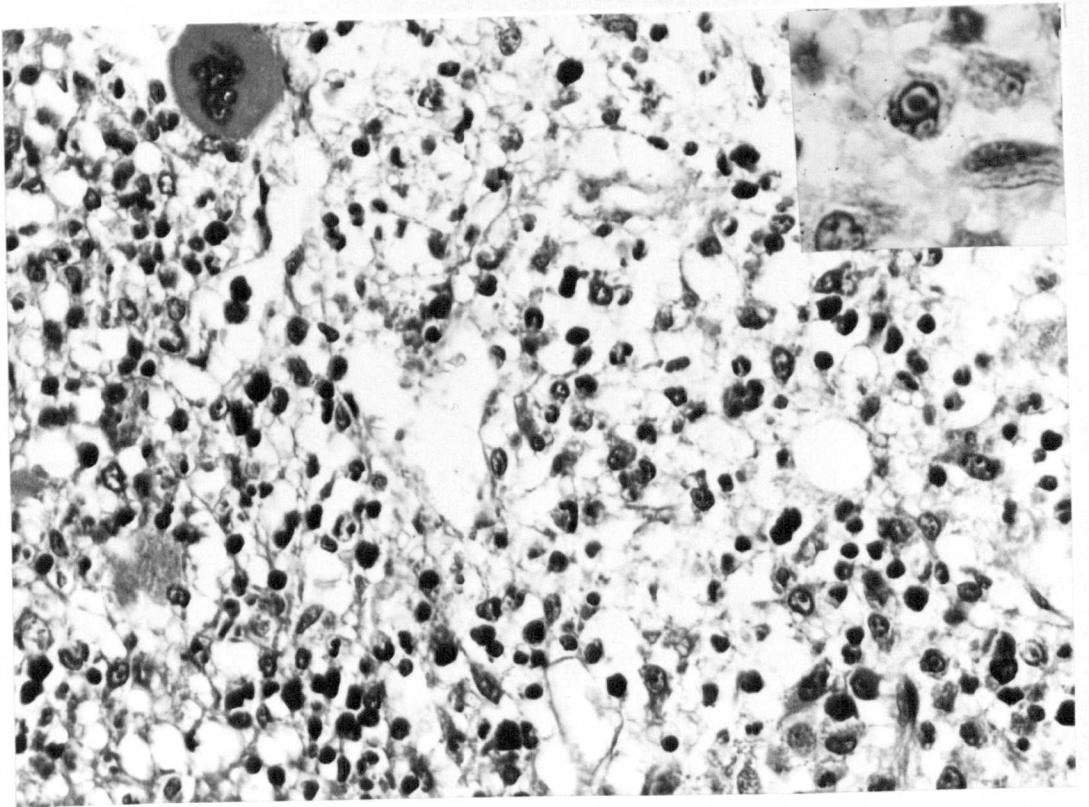
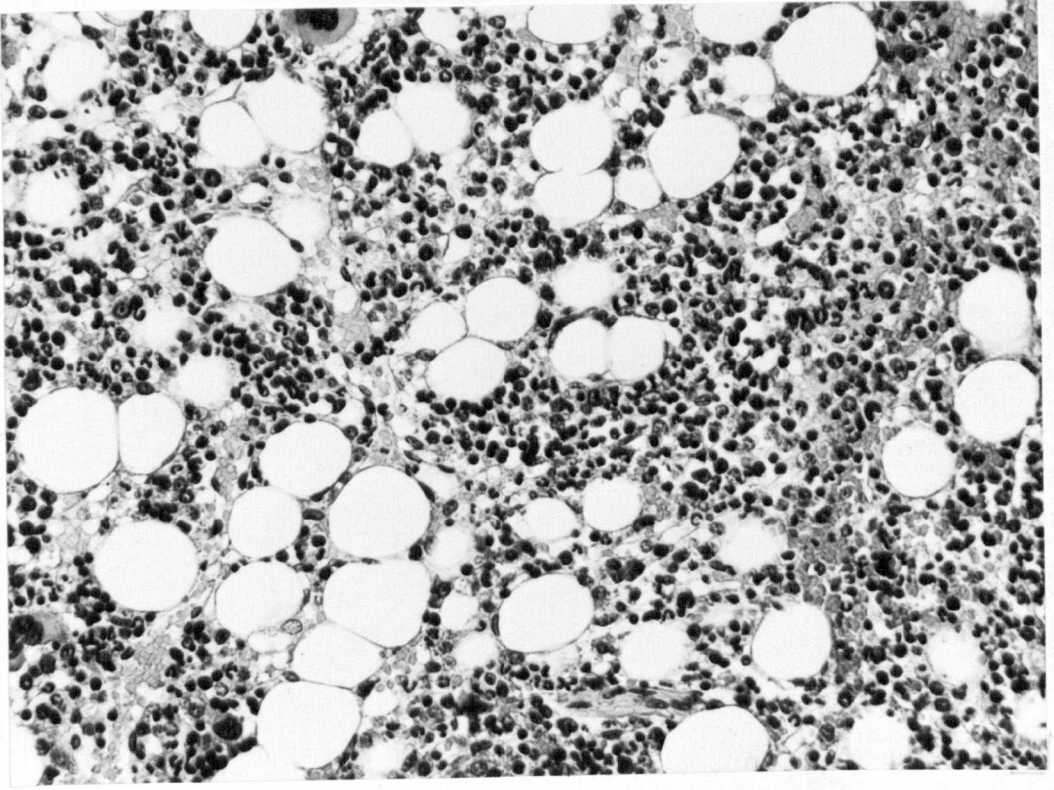
There are numerous mature neutrophils, together with precursor cells of both the myeloid and erythroid series. Control dog.

H & E x 250

Fig. 51: Canine Parvovirus Infection - Marrow of Dog 13.

The marrow is depleted of mature neutrophils. In addition amphophilic intranuclear inclusion bodies may be seen (inset). Taken at 5 DPI.

H & E x 400.
(inset x 1000)



CHAPTER 7 : THE PATHOGENESIS OF CPV ENTERITIS
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INTRODUCTION

Although the experiments detailed in Chapters 3 and 6 above had provided valuable information regarding the clinical features, haematology and pathology of fatal CPV enteritis, in order to establish the definitive pathogenesis of the infection it was still necessary to carry out a sequential study following oral challenge. In particular, it was obviously essential to examine the changes occurring in the first few days after infection and to trace the distribution of antigen in the tissues.

This detailed sequential study was undertaken in the investigation described in this chapter. In addition to the conventional pathological techniques used in Chapters 3 and 6, the immunocytochemical techniques developed in Chapter 5 were applied. These techniques were necessary in order to trace the distribution of virus in tissues throughout the course of infection and to correlate the presence of virus with the development of lesions.

Moreover, scanning electron microscopy (SEM) was used to obtain an alternative view of the changes in the architecture of the intestinal mucosa which are such an important feature of both the natural and the experimental disease.

Prior to the introduction of a commercial scanning electron microscope in 1965, the three dimensional visualisation of mucosal surfaces depended largely on cumbersome reconstructive histological techniques (Cocco et al., 1966) or the use of the dissecting light microscope (Rubin et al., 1960). The former was time-consuming and the latter, though inexpensive, lacked depth of focus and could yield only low magnifications. SEM (Carr, ^{2Tovel}1971) permits detailed study of surface features with both great depth of focus and high magnification.

Jacques and his colleagues (1965) were the first workers to report the application of SEM to the study of intestinal mucosa: he described the mucosal features of normal villi and their epithelial cells in the rat. This study was subsequently expanded by several groups of workers initially using tissue from rats (Carr and Toner, 1968) but later tissue from humans (Marsh and Swift, 1969; Toner and Carr, 1969; Balcerzak et al., 1970).

These workers described the detailed structure of villi in the normal human small intestine. SEM has since been applied to the study of disease syndromes in man using suction biopsy capsules to remove specimens of mucosa from the intestine of conscious patients. For example, the mucosal changes present in coeliac disease have been extensively described (Asquith et al., 1970; Carr and Toner, 1972).

There has been increasing interest in the application of SEM to veterinary diseases. Mebus and Newman (1977) described the SEM features of the intestinal mucosae of calves infected with a reovirus-like agent. They observed shortening of the villi, irregularity of the surface epithelium and, in some places, denudation of the villi. Pearson et al. (1978) used SEM to describe the stereoscopic appearance of the small intestine of a normal calf, and the appearance of the small intestine of a calf with enteric colibacillosis. In the latter animal, villi in the distal small intestine were stunted and there was fusion of the epithelial surfaces. These workers also applied SEM to the study of the small intestine of a calf infected with cryptosporidium, where it permitted the association of the organism with the microvillus brush border in the small intestine to be confirmed (Pearson and Logan 1978). Hutchinson et al. (1980) described the appearance of the small intestine of the cat during infection with Toxoplasma gondii. The villi were slightly shortened in infected animals with parasitised enterocytes appearing as large swollen cells on the surface epithelium.

To date, there has been only one limited report of the application of SEM techniques to the study of the normal canine intestinal mucosa (Hoskins et al., 1982) and no reports of changes observed by SEM in experimental canine intestinal disease. In the study described in this chapter, SEM was used to examine the surface topography of the intestinal mucosa of normal dogs and dogs infected orally with CPV.

MATERIALS AND METHODS

Experimental Animals

The source, housing and maintenance of the experimental animals has been described (Chapter 2). In this experiment, 10 pups, 10 weeks of age were used.

Experimental Design

On their arrival at the laboratory, the dogs were randomly divided into two groups (Table 30). One group of two dogs (25 and 26) was killed at the start of the experiment to monitor health status and to provide non-infected control tissues for histopathological, immunocytochemical and scanning electron microscopical studies. The remaining group of eight dogs (27 to 34) were each orally infected with 1 ml of purified virus suspension of faecal origin with an HA titre of 3.2×10^6 and an infectivity titre of $14.5 \log_{10} \text{TCID}_{50}/\text{ml}$.

Following infection, all dogs were housed separately, examined clinically each day and their rectal temperatures recorded. In addition, daily faecal samples were taken for assay by HA test for viral antigen and HAI test for antibody to CPV. Daily blood samples were collected by jugular venipuncture and were divided for haematological and serological examinations. In addition, serum was examined for the presence of free CPV by virus isolation in FEA cells.

One dog was killed each day from 1 DPI to 8 DPI. A full post-mortem examination was carried out and samples collected for further investigation. The full range of tissues listed in Chapter 2 were removed and blocks fixed in 10% NBF and Bouin's fixative for examination by histological and immunoperoxidase techniques. Duplicate blocks were snap frozen in liquid nitrogen for immunofluorescence examination. Blocks of duodenum, jejunum, ileum and colon were fixed in paraformaldehyde/glutaraldehyde for SEM examination. Samples of intestinal contents were collected for assay of HA and HAI activity. In addition, gastric contents were collected for assay of HA activity and determination of pH.

Procedures Employed

Clinical and post-mortem examinations, haematological, serological, virological, histological and scanning electron microscopical examinations were carried out as detailed in Chapter 2. The immunocytochemical examinations were carried out using the final methods developed in Chapter 5.

RESULTS

Control Dogs

The control dogs were killed at the start of the experiment and were clinically healthy. Rectal temperatures were within the normal range as were the values obtained from haematological examination, which are recorded in Tables 33, 34, and 35. Viral haemagglutinins were not detected in faecal samples from these animals and antibody to CPV was not detected in their sera.

No macroscopical abnormalities were observed at post-mortem examination. The thymic weights and body/thymic weight ratios are recorded in Table 43. Histological examination revealed no abnormalities in the lymphoid or myeloid

tissues. In the intestine, the villi and crypts were well defined. At all levels of the small intestine, the epithelium lining the crypts contained numerous mitotic figures in the proliferative zone, with few mitoses being observed in the basal crypt cells immediately under this area. In the proliferative zone, individual small dark amphophilic cells could be observed either within the epithelium or being extruded from it into the lumen of the crypt. However, these cells were sparsely distributed and were found in only a few crypts. GALT was present throughout the alimentary tract and was particularly prominent in the ileum. Over these areas, the villi were shortened and the crypts were distorted and irregular.

All tissues detailed in Chapter 2 were examined using immunocytochemical techniques. There was no specific staining in any tissue from either of these control animals. Pale yellow autofluorescence was observed in the reticuloendothelial cells of the thymus.

The findings from SEM examination of the intestines of these dogs are described in detail below.

Infected Dogs

Clinical Signs

The clinical signs are shown in Table 31. Clinical abnormalities were first noted at 4 DPI when dog 33 was slightly duller than normal and had a reduced appetite, although some solid food was still taken. By 5 DPI, dog 33, together with dogs 32 and 34 were dull, completely anorexic and passing soft, diarrhoeic faeces. Some blood was present in the stool of dog 32. Also on 5 DPI, dog 31 was dull and anorexic; no faeces were produced by this dog on this day.

Dog 32 deteriorated rapidly overnight, and on 6 DPI was prostrate, dehydrated and passing profuse dysenteric faeces.

This animal was killed on the morning of 6 DPI. Dog 33 at 6 DPI was still dull and anorexic with mucoid diarrhoea; there was moderate dehydration with apparent weight loss. Dog 34 was brighter at 6 DPI than it had been at 5 DPI, but was still anorexic.

At 7 DPI, dog 33 was still dull and anorexic, though no longer diarrhoeic and dog 34 appeared to have recovered and was killed at 8 DPI.

There was no elevation of rectal temperatures (Table 32) above the normal range in any dog in this study. Vomiting was not observed.

Haematological Findings

Absolute values for the total leukocyte counts, neutrophil and lymphocyte counts are shown in Tables 33, 34 and 35.

Changes in the haematological patterns of the infected dogs were first seen at 3 DPI, when there was a slight ^{RELATIVE} reduction in the absolute lymphocyte counts in four of the six remaining dogs. By 4 DPI, this reduction in lymphocyte counts was more marked, with an absolute lymphopaenia in one, dog 33 (where the lymphocyte count was $0.6 \times 10^9/l$). In no dog on either 3 or 4 DPI was the relative or absolute lymphopaenia reflected in a significant reduction in the total circulating leukocyte counts.

At 5 DPI, there was a reduction in total leukocyte counts in dogs 32 and 33, due to an ^{ABSOLUTE} reduction in both neutrophils and lymphocytes, with a relative lymphopaenia and neutropaenia in both dogs. This reduction was maintained until they were killed at 6 and 7 DPI respectively.

A similar, although less marked, reduction in the total leukocyte count in dog 34 was seen at 6, 7 and 8 DPI.

Virological Findings

Virus isolation was carried out on the daily serum samples collected from each dog. The results are shown in Table 36. Virus was isolated from the sera of all dogs at 3 and 4 DPI although the sample size was insufficient to allow titration of the exact amount present.

Daily faecal specimens were examined for the presence of viral haemagglutinin. Faecal haemagglutinin was first detected at 3 DPI in dog 32 at a titre of 16 (Table 37). By 4 DPI, haemagglutinin was present in four of the five remaining dogs. Very high titres of 4096 were recorded from dogs 30, 32 and 33. A lower titre was present in the sample from dog 31. No haemagglutinin was detected in the faeces of dog 34. At 5 DPI, haemagglutinin was not detected in the faeces of dog 32, although there were levels of 4096 in the faeces of three remaining animals. The faeces of dog 32 were again negative at 6 DPI, while those of the two remaining dogs, 33 and 34, again contained large amounts of specific haemagglutinin. No faecal specimen was obtained from dog 33 on 7 DPI but a titre of 4096 was found in the faeces of dog 34. On 8 DPI, the sample from dog 34 was negative.

Samples of small intestinal and gastric contents from each dog were collected post-mortem and examined by HA test. The results are shown in Table 38. Haemagglutinin was first detected in intestinal content of dog 30 killed at 4 DPI at a titre of 4096. A similar titre was present in the content of dog 31, killed at 5 DPI but no haemagglutinin was found in dogs killed later and electron microscopy of these later samples failed to reveal virus particles. Haemagglutinin was recorded in the gastric content of only one dog, dog 31 killed at 5 DPI; the gastric contents of this dog had been noticeably bile-stained.

Serological Findings

The results of antibody analysis of daily serum samples are shown in Table 39. Antibody was first detected in the serum of all dogs remaining at 5 DPI at titres from 8 to 64. The titres rose thereafter with levels of 2048 or greater by 7 DPI.

Daily faecal specimens were also examined by HAI test for the presence of antibody (Table 40). Specific antibody to CPV was found in only one sample, from dog 34 at 8 DPI when a titre of 32 was recorded.

Intestinal and gastric contents were also examined by the HAI test for the presence of antibody (Table 41). Intestinal antibody was detected in dogs 33 and 34 at 7 and 8 DPI. Antibody was not detected in the gastric contents.

Pathological Findings

Macroscopical Findings

The findings at post-mortem examination are summarised in Table 42.

No abnormalities were detected in the dogs killed at 1 and 2 DPI and only slight oedema of the thymus in dog 29, killed at 3 DPI. In dog 30, killed at 4 DPI, there was petechiation of the anterior lobe of the thymus and extensive thymic oedema. The severe oedema was reflected in a reduction in body/thymic weight ratio (Table 43). In dogs killed from 5 DPI onwards, there was an obvious reduction in thymic mass. The thymus was small but recognisable at 5 DPI, but only a thin, lacy remnant at 6, 7 and 8 DPI. Peripheral lymph nodes were enlarged and slightly oedematous in all dogs killed after 4 DPI, with the exception of the mesenteric nodes in dogs 32, 33 and 34 which were small.

Macroscopic lesions were found in the intestines only of dogs 32 and 33 killed at 6 and 7 DPI. In dog 32, the wall of the duodenum was thickened and the mucosa was haemorrhagic. In dog 33, the wall of the jejunum and duodenum was thickened and oedematous. Serosal granularity was not seen.

Abnormalities were noted in the stomachs of only two dogs. In dog 31, killed at 5 DPI, the scanty mucoid contents were bile-stained. In dog 32, killed at 6 DPI, a clear grey mucoid fluid was present. The pH of the gastric contents in dog 32 was 8.5. In all other dogs, the pH was less than 5.

Microscopical Findings

All tissues detailed in Chapter 2 were examined. Changes were found only in the lymphoid tissues, intestines and bone marrow. To facilitate description, the alterations will be described on a sequential basis for each organ.

Thymus

Lymphocytolysis and depletion of cortical thymocytes were the predominant features in this tissue (Table 44). Active lymphocytolysis was first noted at 2 DPI and was more widespread at 3 and 4 DPI. Interstitial oedema was also a feature on these days, being most extensive in dog 30, killed at 4 DPI which had had the decreased body/thymic weight ratio. In dog 31, killed at 5 DPI, depletion of cortical thymocytes was the predominant finding, with little active cellular destruction or interstitial oedema. There was also a degree of depletion of medullary thymocytes. Depletion of the thymic cortex was the major feature in all dogs killed after 5 DPI and was most pronounced in dog 32, killed at 6 DPI while showing clinical signs of disease.

Carcase and visceral lymph nodes

Lymphocytolysis and depletion were again the predominant changes in the nodes (Table 44). The earliest lymphocytolytic changes were noted in dog 28, killed at 2 DPI. In dog 29, examined at 3 DPI, darkly staining amphophilic cells were seen in the germinal centres; the tonsils seemed most severely affected. At 4 DPI, there was widespread lymphocytolysis in all nodes with pyknosis, karyorrhexis and severe lymphoid depletion in the germinal centres. In the dogs killed at 5 and 6 DPI, the germinal centres were reduced to their histiocytic reticulum cell supporting framework. Depletion of the paracortex and the medullary cords was also noted in these dogs but there was little evidence of active cellular destruction.

In dog 33, killed at 7 DPI, depletion was still the predominant feature but there was repopulation of the germinal centres with lymphoid cells, together with some expansion of the paracortex. Regeneration and repopulation of the lymph nodes was much more advanced in the dog killed at 8 DPI.

Spleen

Changes in the spleen paralleled those in the lymph nodes with early lymphocytolysis at 2 DPI progressing to depletion at 5 and 6 DPI with expansion of the splenic white pulp at 8 DPI.

Alimentary tract

No destructive or inflammatory changes were found in the stomach of any infected dog. In dogs 27, 28 and 29, killed at 1, 2 and 3 DPI, no alterations in intestinal architecture were apparent. (Table 45). Small dark amphophilic cells were noted occasionally in the intestinal crypts but these were not present in greater number than in the control dogs. No abnormalities were noted in the caecum or colon but lympho-

cytolysis of a similar pattern to that in the lymph nodes was present in the GALT at 3 DPI.

The first abnormalities in the small intestinal epithelium were noted in dog 30, at 4 DPI, when changes of histological pattern A were present at all levels of the small intestine. These changes were most extensive in the duodenum, and became more focal in distribution distally. No abnormalities were found in the caecum and colon but once again there was lymphocytolysis in the GALT.

At 5 DPI, in dog 31, changes of pattern A were again found and were most extensive in the duodenum. In the ileum, the amphophilic cells were particularly numerous in the crypt epithelium immediately overlying or adjacent to the GALT and were more focally distributed elsewhere. In the GALT itself there was active lymphocytolysis and numerous pale staining intranuclear inclusion bodies were found. The caecum and colon appeared normal.

At 6 DPI, in dog 32, extensive changes of pattern B were present in the duodenum. Both attenuation of epithelial cells and dilation of crypts were evident in the duodenum but in the jejunum only attenuation was present and lesions were much more focally distributed. Again, particularly in the duodenum, the epithelium at the tips of the shortened villi was often vacuolated and in some cases appeared separated from the underlying lamina propria. Obvious intranuclear inclusion bodies were present in attenuated epithelial cells in both duodenum and jejunum and were particularly noticeable in sections from tissues fixed in Bouin's. In the ileum, pattern A was still present. At all sites, increased numbers of neutrophils were evident in the intestinal lumen in a mucoid exudate. The GALT was severely depleted and foci of neutrophils were present in the germinal centres. Neutrophils were not evident in other areas of the lamina propria or submucosa. Amphophilic cells were present in increased numbers in the

epithelium of the caecum and colon.

In dog 33, killed at 7 DPI, regenerative lesions of pattern D were present throughout the small intestine. Only regenerative changes were noted in the duodenum but in the jejunum some epithelial attenuation and crypt dilation were still apparent. In some areas, a regenerative change was evident towards the base of the crypt/villus units while attenuation and dilation was obvious in the epithelium above this. Similarly in the ileum, a mixture of patterns, with A in some areas, and D in others was found. A feature of the regenerative areas, particularly in the ileum, was the presence of an increased number of goblet cells in the epithelium lining regenerating crypts and their associated villi. In the GALT, depletion without active lymphocytolysis was present. Early repopulation of the germinal centres was found in some areas. Increased numbers of amphophilic cells were again present in the epithelium of the caecum and colon.

By 8 DPI, in dog 34, regenerative changes of pattern D were present in all regions of the small intestine. Repopulation with some expansion of the GALT was noted. The caecum and colon appeared normal.

Bone marrow

Changes in the bone marrow were noted only in dog 32, killed at 6 DPI, during the phase of clinical illness. The pool of mature neutrophils in the marrow was depleted.

Immunocytochemical Findings

Both immunofluorescence and immunoperoxidase techniques were used. Immunofluorescence was used as an initial screen on all tissues examined: submandibular, retropharyngeal, mesenteric and popliteal lymph nodes, thymus, palatine tonsil, spleen, lung, heart, liver, kidney, bone marrow, stomach,

duodenum, jejunum, ileum, caecum and colon. Immunoperoxidase was then applied to specific tissues to permit optimal definition and more specific localisation of antigen. Tissues examined by PAP were duodenum, jejunum, ileum, caecum, colon, mesenteric, retropharyngeal and popliteal lymph nodes, thymus and bone marrow. Where stated, sections of liver were also examined by PAP.

The results of the immunocytochemical examinations are summarised in Table 46.

Antigen was first detected in the thymus of dog 27, killed at 1 DPI, where individual positively stained cells were observed in the thymic cortex (Fig. 52). In dog 28, killed at 2 DPI, there were again positive cells in the thymic cortex, which were slightly more numerous than in dog 27. The antigen in both these dogs was within the nuclei of thymocytes and was accurately localised and defined using the PAP method. In addition, in dog 28, there was specific fluorescence in the germinal centres of the retropharyngeal lymph node. This was limited to a few cells, which on PAP examination appeared to be centroblasts. The antigen was intranuclear.

In dog 29, killed at 3 DPI, the thymus, GALT, submandibular and mesenteric lymph nodes, together with the spleen, were positive for viral antigen. In the thymus, antigen was in the nuclei of cortical thymocytes. Similarly, in the lymph nodes and GALT, antigen was localised to the germinal centres and was within the nuclei of centrocytes and centroblasts (Fig. 53, 54). Splenic antigen was limited to the white pulp where individual cells were positive.

There was extensive nuclear fluorescence in the cortex of the thymus of dog 30, at 4 DPI (Fig. 55). This was confirmed using the PAP technique which also demonstrated some antigen taking into the intercellular connective tissue (Fig. 56). In addition, there were focal positive cells in the germinal

centres of all lymph nodes and GALT examined; antigen was within the nuclei of centroblasts. For the first time, antigen was detected in the intestines with numerous positive cells in the proliferative epithelium of the crypts of the duodenum, jejunum and ileum. Fluorescence in these cells appeared to be focal and intranuclear (Fig. 57, 59), although it was difficult to determine the morphology of the cells containing the antigen. On examination using the PAP technique, these cells were identified as the small dark amphophilic cells which had been noted in conventional histological preparations (Fig. 58, 60). Antigen was not detected in the caecum or colon of this dog using either technique.

Specific staining in the thymus was less of a feature of dog 31, killed at 5 DPI. Staining by both immunofluorescence and PAP was weak and antigen was diffusely distributed throughout the intercellular connective tissue. The lymph nodes, spleen and GALT in this dog were all positive for viral antigen, both in the nuclei of centrocytes and taking into the intercellular connective tissue (Fig. 61, 62). In the tonsil and the retropharyngeal lymph node, antigen was present, not only in the germinal centres, but also in the paracortex and medullary cords although here it was completely extracellular. There was again specific staining of the amphophilic cells of the proliferative area of the crypts in the small intestine. More positive cells were detected in the duodenum than in the jejunum and ileum. In addition, in the caecum, there was positive staining of cells in the crypt epithelium overlying the GALT. Elsewhere in the caecum and colon, antigen could not be detected. There was specific staining of one cell in the bone marrow of this dog using immunofluorescence but positive staining could not be detected using the PAP method. Weakly positive cells were seen in the liver of this dog and, on PAP examination, these were identified as Von Kupffer cells; antigen was present only in the cytoplasm (Fig. 63).

In dog 32, killed at 6 DPI, there was a small amount of positive staining in the thymic cortex using both immunofluorescence and PAP; antigen was predominantly in the extracellular spaces. Slightly more antigen was located in the germinal centres of the lymph nodes, GALT and splenic white pulp, but it was again predominantly extracellular (Fig. 64). There was focal specific staining in the bone marrow (Fig. 65, 66), with antigen in the nuclei of medium sized basophilic cells, which could not be definitely identified. Detailed morphological identification of the cells was difficult, even using the PAP technique, since the brown stain tended to overlie and obscure nuclear morphology. Antigen was present in the crypt epithelium at all levels of the small intestine and large intestine. Again, this epithelial antigen was contained in the nuclei of the small dark amphophilic cells but there were large amounts of antigen along with cellular debris in the lumina of dilated crypts (Fig. 67, 68). There was specific staining in the cytoplasm of Von Kupffer cells in the liver.

In dog 33, killed at 7 DPI, there was little antigen in the thymic cortex and only slightly more in the germinal centres of the lymph nodes, GALT and spleen. In all the lymphoid tissues, antigen was not present in the lymphocytes themselves but in the intercellular connective tissue (Fig. 69, 70). There was antigen in the crypt epithelium at all levels of the intestine. The number of positive cells increased distally to a maximum in the ileum and colon. Antigen was again associated with the dark amphophilic cells, although there were some larger cells with positively stained intranuclear inclusion bodies.

In dog 34, killed at 8 DPI, only a trace of intercellular antigen was located in the lymphoid tissues. There was no specific fluorescence in the crypt epithelium at any region of the intestine.

Scanning Electron Microscopical Findings

Control Dogs

In the control dogs, the majority of villi in the small intestine, irrespective of site, were finger-like in shape (Fig. 71) although occasional leaf-shaped villi were seen in the duodenum and jejunum. It was impossible to estimate accurately the length of the villi but at all sites they were of uniform height. The breadth of villi did vary, with the broadest in the duodenum (Fig. 71) and the most slender in the ileum (Fig. 72). Strands of mucus were found over and between the villi (Fig. 72) but these were generally tenuous and only occasionally obscured the surface.

On detailed examination of individual villi, three surface features were observed. At low magnifications, numerous shallow, irregular grooves were noted running transversely around the villi along their length (Fig. 72). The second and third features, noted on higher magnifications, were outlining of the epithelial cells (Fig. 73) to give a mosaic effect and the presence of numerous pits in the epithelial surface (Fig. 74). Between these outlines of individual epithelial cells, the surfaces were slightly domed and had a velvety appearance, presumably due to the dense pile of microvilli (Fig. 73). Doming became more marked towards the tips of the villi and, at the apices, cells were separated, rounded and had lost their velvety pile (Fig. 73). Some of the pits on the surface of the villi contained a central elevation. Pits were interpreted as being goblet cells and the elevated central mass as discharging mucus. In some specimens, the circumvillar basins with crypt mouths opening into them could be discerned (Fig. 75).

Villi were not present in the colon. The surface of the colonic mucosa consisted of a series of openings, regularly arranged (Fig. 76). Mucus and debris was seen in some of

these crypt openings.

Infected Dogs

No alterations in intestinal surface morphology were observed by SEM in dogs 27-31 killed from 1-5 DPI.

Changes were first seen in dog 32, killed at 6 DPI, while showing clinical signs of enteric disease. In this animal, the most prominent finding was a thick sheet of mucus covering the mucosal surface at all levels of the intestine (Fig. 77). Details of mucosal structure could only be observed through the relatively infrequent breaks in this mucous blanket but nonetheless alterations were noted at all levels of the small intestine.

In the duodenum, the villi were irregular in height but generally shortened (Fig. 78). The villi also appeared thickened when compared to controls and in some areas there was apparent epithelial fusion of adjacent villi. There was complete loss of the irregular transverse grooves around the villi. Cells towards the apices of the villi were swollen and in some cases were missing (Fig. 79) exposing the underlying lamina propria. The circumvillar basins at the base of the villi appeared widened and crypt mouths dilated. Numerous small round cells, probably neutrophils or possibly altered red cells, together with a few typical indented erythrocytes, were present over the exposed lamina propria, scattered on the surface of the epithelial cells and on, or embedded in, the mucous coat. In addition, larger rounded epithelial cells and associated small strips or sheets of detached epithelial cells were present in the surface debris.

In the jejunum, lesions were more focal than in the duodenum, but, where present, shortening of the villi was the most striking finding. The villi were reduced to stubby projections with complete loss of the transverse grooves. In

severely affected areas, the stubby villi were denuded of their epithelium on some aspects while other areas of the same villi were covered by an intact, although swollen, epithelium (Fig. 80). Numerous neutrophils, erythrocytes and detached epithelial cells were observed over and around affected areas.

In the ileum, shortening of the villi was again present, although it was less extensive than in the higher regions of the intestines; leaf-shaped villi were apparent (Fig. 81). The transverse grooves around the villi were still present. The goblet cell pits on the surface of the villi appeared to be increased in number and most were discharged with only a few showing central projections of mucus.

No changes were observed in the colon of this animal.

In dog 33, killed at 7 DPI, a thick blanket of mucus was again the major feature noted. In this dog, the blanket was so extensive and continuous in the duodenum and ileum that despite examination of numerous blocks details of the underlying mucosal surface were not observed. Mucus was also present over the jejunum but at this site gaps were found and examination of the surface structure was possible. The villi were short and conical in shape (Fig. 82). Some villi appeared to be fused at their bases (Fig. 82) although this appearance may equally have been the result of hypertrophy of the intervillous ridges. In other areas there was apparent fusion towards the tips of the shortened villi (Fig. 83). Transverse grooves were evident on the surface of the villi except where there was high fusion. No changes were observed on the colonic mucosa.

In dog 34, killed at 8 DPI, only thin strands of mucus were present and the mucosal structure was easily observed. In the duodenum, the villi were once again finger-like in shape although there was variation in height (Fig. 84). In addition, the apices of the villi were irregular in shape; some were noticeably pointed (Fig. 84) and similar to the short conical

villi found in the jejunum of dog 33 at 7 DPI. In the jejunum at 8 DPI, the villi, though taller than at 7 DPI, were still noticeably short and conical. The sections of ileum from this dog were accidentally damaged in processing and were not examined. No abnormalities were noted in the colon.

DISCUSSION

It was the aim of this experiment to investigate the detailed sequential changes following oral infection with CPV, to trace the localisation of viral antigens in various tissues and to correlate these with the development of clinical disease. The numerous parameters examined in this study permitted these aims to be fulfilled.

As in Section 6, clinical signs first became apparent at 4 DPI with dullness and anorexia the earliest features noted. By 5 DPI, evidence of primary enteric disease in the form of diarrhoea was present in all 3 remaining dogs. As in Section 6, the "take" of illness was high with all dogs remaining alive when clinical illness might be expected being affected although only one of the three developed dysentery. Vomiting was not recorded in this experiment but this may represent, in part, a lack of observation. Intermittent vomiting may not have been observed and any vomitus would have been adsorbed by the bedding within a very short period.

The haematological picture was **generally** that of a **relative** lymphopaenia from 3 DPI which would not have been noted had only total leukocyte counts been monitored. Reduction in total leukocyte counts occurred only during the phase of clinical enteric illness when it was associated with a relative lymphopaenia and a neutropaenia. Viral antigen was first detected at high titre in faeces and intestinal contents at 4 DPI. Serum antibody first appeared at 5 DPI and was first recorded in intestinal contents at 7 DPI. The rapid rise in

serum antibody and the apparent leak into the intestinal lumen (or production of local antibody) coincided with diminution and cessation of detectable excretion of virus. The only apparent anomaly in these results was the group of results for dog 32, killed while dysenteric at 6 DPI. In this dog neither viral antigen nor specific antibody was demonstrated in faeces or intestinal contents.

The failure to demonstrate free viral antigen in faeces or contents is not, in itself, surprising since this animal was dysenteric and serum antibody, present at a titre of 256, might have been expected to have complexed any virus particles present. However, no residual free antibody was detected in faeces or contents. Total complexing of viral antigen and specific antibody is an attractive explanation for these results but unfortunately complexed viral arrays were not detected by transmission electron microscopy of negatively stained faecal and content preparations. Subsequently, immunocytochemistry demonstrated that viral antigens were indeed present in the intestinal epithelia of this dog and in view of this it is difficult to explain the inability to detect either free virus, free antibody or complexes in faeces and intestinal contents. The simplest explanation may be that one of the results was wrong.

As in the previous studies, pathological changes were confined to the lymphoid and alimentary systems and the changes in this study were similar in type to those recorded in Chapters 3 and 6. It was of interest that in the dog with excessive mucoid fluid in the stomach, the pH (8.5) was alkaline indicating that this could well have been of salivary origin.

The sequential nature of this study revealed the initial changes to be in the lymphoid tissues with lytic change first apparent in the thymus at 2 DPI and generalised lymphocytolysis by 3 DPI. Intestinal lesions were not noted until 4 DPI and despite the presence of severe destructive

changes in the GALT, the intestinal epithelial lesions were not solely related to the presence or absence of underlying lymphoid tissues. As in Chapter 6, changes were more extensive and progressed more rapidly in the upper small intestine than in the ileum. Again, lesions were not found in the stomach and changes in the large intestine were minimal.

Changes in the thymus were once more the most consistent finding. Marked thymic oedema led to a decrease in the body/thymic weight ratio at 4 DPI but thereafter a significant increase in this ratio was found in all dogs.

The most important results of this study were those obtained from the application of immunocytochemical methods. These specialised staining techniques revealed that antigen was first present in the thymus at 1 DPI, had reached the rest of the lymphoid tissues by 2 DPI and first appeared in the small intestine at 4 DPI. The development of histological lesions in the lymphoid tissues and intestinal epithelia therefore follows closely on localisation and replication of virus in these tissues. Immunocytochemical techniques confirmed that viral localisation and replication in the intestine was limited to the proliferative zone of the crypt epithelium and that the increased numbers of small amphophilic cells observed by conventional microscopy were the result of viral replication and cell death.

An unexpected finding using immunocytochemical techniques was the presence of specific staining in the liver. This was unexpected since the liver in the weaned pup shows little in the way of mitotic activity and would therefore seem unlikely to be a preferred site for viral localisation and replication. However, parvoviruses have been isolated from the livers of cats and dogs experimentally and naturally infected with, respectively, FPV and CPV (Csiza et al., 1971a and b; Azetaka et al., 1981). The immunocytochemical studies in this experiment demonstrated that viral antigen in the liver was

localised specifically to the cytoplasm of Kupffer cells lining hepatic sinusoids at 5 and 6 DPI when there was maximal amounts of viral antigen in the small intestine and a developing humoral immune response. The nuclei of the Kupffer cells and other hepatic cell types were uniformly negative. These findings indicate that the hepatic antigen is not a reflection of primary viral replication in the liver tissues. It may be the result either of phagocytosis and sequestration of virus originating in the intestine and reaching the liver via the portal system or, alternatively, of uptake of immune complexes from the general circulation.

The use of SEM techniques allowed an alternative method of visualising mucosal damage following CPV infection. The surface morphology in the control dogs was similar to that described by Hoskins et al. (1982) in the dog, Pearson et al. (1978) in the calf, Waxler (1972) in the pig and Toner and Carr (1969) in the human small intestine. In the infected dogs, alterations in surface topography were not observed until 6 DPI. Conventional histological preparations had revealed changes in the intestinal crypts at 4 and 5 DPI but alterations in the villi, basically shortening, were slight and obviously insufficiently severe to produce recognisable alterations in surface morphology by SEM. However, it is possible, although unlikely, that the area of intestine selected for SEM examination was unaffected.

The mucosal changes at 6 DPI were shortening and thickening of the villi with loss of their transverse grooves, disruption of cellular outlines and areas of epithelial loss around the apical extrusion zone. The short pointed villi at 7 DPI appeared to be regenerative, based on the observation of hypertrophy of intervillus ridges and prominent transverse grooves on the villi. The long slender villi seen at 8 DPI, with short pointed tips, would further suggest that the changes seen at 7 DPI were regenerative, although conclusive evidence of regeneration depends on histological findings.

Shortening of villi has also been reported in reovirus infection (Mebus and Newman, 1977) and enteric colibacillosis (Pearson et al., 1978) in the calf. However, a significant

feature of these infections was preservation of the surface features despite shortening of the villi. Marked stunting of villi was also observed in pigs infected with the coronavirus of transmissible gastroenteritis (Waxler, 1972). Surface grooves were present except in the most extreme cases where villi were reduced to short knobs on the mucosa with few remaining features. However, the significant differences from the present study were the maintenance of epithelial confluency and the absence of dilation of crypt mouths and circumvillar basins.

In the mouse, following acute irradiation, Carr and Toner (1972) observed changes very similar to those in this study. Villi were reduced to small "wartlike excrescences" with complete loss of the regular surface grooves of normal animals. Further, again concurring with this study, there was dilation of crypt mouths and circumvillar basins.

The preservation of surface features on the villi in reo and coronavirus infections and their absence in CPV infection and irradiation damage appears to be a further illustration of the differing pathogeneses of these conditions. Reo and coronavirus infect cells at the tips of villi, effectively increase cell loss and, in the face of a failure of immediate replacement, there is shortening of villi. Irradiation damage and CPV infection also result in a net loss of cells although, in contrast, this is due to destruction of precursors in the proliferative area of the crypt epithelium.

Loss of cells from the proliferative zone would lead to most of the alterations observed in this study. Shortening of villi would reduce the total surface to be clothed from the damaged proliferative zone and loss of the transverse grooves could be the result of the available epithelium effectively "stretching" over the lamina propria in an attempt to maintain epithelial integrity. Since a continuous supply of mature cells would no longer be available, effete cells at the apices of the villi would be less likely to be extruded by pressure from

advancing cells. The rounded cells visualised at the tips of the villi by SEM at 6 DPI would therefore seem likely to represent an increased area of non-extruded effete cells corresponding to the vacuolated epithelial cells, observed by conventional histological techniques. Lifting of these vacuolated cells from the lamina propria was also noted on conventional histological examination; stripping of epithelial cells from the tips of villi seen by SEM may simply represent artefactual detachment of these lifted, effete cells during processing.

Two other features of the SEM results are worthy of discussion. First, the thick, extensive blanket of mucus found at 6 and 7 DPI appeared to be an integral part of the disease process, corresponding to the mucus observed by conventional microscopy at these times. The presence of the mucus did make observation of the mucosa difficult but attempts to remove the mucous coat were unsuccessful (unpublished observations) and would have been likely to have increased artefactual damage. Second, visualisation of lesions below the level of the villi was limited although dilation of crypt mouths and widening of circumvillar basins were appreciable. Cryofracture procedures would have permitted more detailed 3 dimensional examination of the crypt lesions but were not attempted in this study.

The results obtained from this detailed sequential study have allowed the formulation of the detailed pathogenesis for CPV infection in the dog. Following oral infection, the initial site of viral replication is in the thymus (from 1 DPI) rapidly followed by the other lymphoid tissues (from 2 DPI). Viral replication in these organs results in lymphocytolysis and depletion. The final link in the pathogenesis is provided by the detection of a plasma viraemia in infected dogs at 3 and 4 DPI, presumably the result of release of virus into the circulation from the lymphoid tissues. This viraemia permits the localisation of virus in the mitotically active proliferative zones of the intestinal epithelia from 4 DPI.

Destruction of epithelial cells in these areas results in excretion of virus in faeces and may cause sufficient derangement of intestinal structure to result in clinical signs of enteric disease. Coincident with these events is the development of a rapid humoral immune response (from 5 DPI) and as serum antibody rises so virus is eliminated from the body.

The results of these studies, together with those of other workers (Appel et al., 1979a; Robinson et al., 1980b; McAdaragh et al., 1982; Pollock, 1982) indicate that the initial phase of CPV infection i.e. replication of virus in lymphoid tissues, always occurs in the non-immune host. The variable component of the infection is the degree of damage to intestinal architecture which results from localisation of virus in small intestinal crypts and which, in turn, dictates the severity of the clinical syndrome.

The crucial factor influencing the extent of intestinal lesions would seem to be the mitotic activity in the proliferative zone of the intestinal crypts at the time (3-4 DPI) when viral localisation at this site occurs. If many mitoses are present, many cells will support replication of virus and extensive damage with severe disease will develop. If mitoses are few, only limited replication with minor pathology and transient illness or asymptomatic viral excretion will result.

The numerous factors which might affect the mitotic rate in the intestinal crypts and which might therefore contribute to the development of affection as opposed to infection with CPV are discussed in detail in the following chapter.

TABLE 30.

CHAPTER 7 : EXPERIMENTAL DESIGN.

	Dog Number	Day Killed
Control Group	25	0
	26	0
Infected Group	27	1
	28	2
	29	3
	30	4
	31	5
	32	6
	33	7
	34	8

TABLE 31.

CHAPTER 7 : CLINICAL SIGNS IN EXPERIMENTAL DOGS

Dog	0	1	2	3	4	5	6	7	8
25	-								
26	-								
27	-	-							
28	-	-	-						
29	-	-	-	-					
30	-	-	-	-	-				
31	-	-	-	-	-	A+			
32	-	-	-	-	-	A++	A+++		
						D++	D+++		
33	-	-	-	-	A+	A++	A++	A+	
						D+	D+		
34	-	-	-	-	-	A++	A+	-	-
						D++			

A = Dullness and Anorexia. D = Diarrhoea. + = Mucoïd. ++ = Fluid. +++ = Dysenteric. V = Vomition.
+ to +++ on severity.

TABLE 32.

CHAPTER 7 : RECTAL TEMPERATURES (°C)

Dog No.	0	1	2	3	4	5	6	7	8
25	38.3								
26	38.1								
27	38.2	38.5							
28	38.5	38.6	38.3						
29	38.2	38.7	38.2	37.7					
30	37.3	39.5	38.5	38.9	39.5				
31	38.2	38.5	38.3	38.3	38.2	38.0			
32	38.4	38.5	38.3	38.6	38.8	39.0	38.8		
33	38.0	38.3	38.5	38.3	39.5	36.8	38.3	37.4	
34	38.4	38.4	38.5	38.4	38.7	38.7	38.9	38.2	37.3

TABLE 33.

CHAPTER 7 : TOTAL CIRCULATING LEUKOCYTE COUNTS (x 10⁹/Litre)

Dog No.	Day Post Infection								
	0	1	2	3	4	5	6	7	8
25	18.0								
26	28.5								
27	21.0	8.7							
28	16.1	17.8	15.3						
29	20.8	18.7	19.5	20.9					
30	20.7	17.7	17.4	35.3	18.0				
31	12.5	16.0	14.0	13.9	12.3	14.8			
32	17.6	19.1	17.6	17.4	19.5	9.7	9.5		
33	15.3	16.3	16.5	15.6	30.6	8.5	9.5	7.7	
34	23.9	22.3	21.5	18.2	31.1	24.5	16.7	18.0	12.3

TABLE 34.

CHAPTER 7 : ABSOLUTE CIRCULATING NEUTROPHIL COUNTS ($\times 10^9/\text{Litre}$)

Dog No.	Day Post Infection								
	0	1	2	3	4	5	6	7	8
25	15.9								
26	22.8								
27	17.6	4.6							
28	14.4	15.0	11.4						
29	19.1	16.7	16.8	17.9					
30	14.1	13.7	12.5	32.1	16.6				
31	10.3	12.3	10.2	11.8	10.9	13.6			
32	14.25	15.6	12.4	13.9	16.8	8.0	7.8		
33	11.3	10.75	11.1	13.1	30.0	6.2	7.8	5.0	
34	20.0	17.2	15.15	14.8	29.5	20.0	13.8	15.5	8.4

TABLE 35

CHAPTER 7 : ABSOLUTE CIRCULATING LYMPHOCYTE COUNTS ($\times 10^9/\text{Litre}$)

Dog No.	Day Post Infection							
	0	1	2	3	4	5	6	7
25	1.9							
26	5.3							
27	3.15	4.1						
28	1.7	3.0	3.9					
29	1.56	2.4	2.7	3.0				
30	2.3	4.0	4.9	3.2	1.4			
31	2.45	3.6	3.8	2.1	1.35	1.2		
32	3.3	3.5	5.2	3.5	2.7	1.7	1.7	
33	4.0	5.5	5.2	2.5	0.6	2.3	1.8	2.7
34	4.0	5.1	6.3	3.4	1.6	4.5	2.9	2.5
								3.9

TABLE 36.

CHAPTER 7 : EXAMINATION OF SERA BY VIRUS ISOLATION
FOR THE PRESENCE OF CANINE PARVOVIRUS.

Dog No.	Day Post Infection								
	0	1	2	3	4	5	6	7	8
25	-								
26	-								
27	-	-							
28	-	-	-						
29	-	-	-	+					
30	-	-	-	+	+				
31	-	-	-	+	+	-			
32	-	-	-	+	+	-	-		
33	-	-	-	+	+	-	-	-	
34	-	-	-	+	+	-	-	-	-

TABLE 37.

CHAPTER 7 : EXAMINATION OF FAECAL SPECIMENS FOR THE PRESENCE OF SPECIFIC CPV HAEMAGGLUTININ*

Dog No.	0	1	2	3	4	5	6	7	8
25	ND								
26	ND								
27	ND	< 2							
28	< 2	< 2.	< 2						
29	< 2	< 2	< 2	< 2					
30	ND	< 2	< 2	< 2	4096				
31	< 2	< 2	< 2	< 2	32	4096			
32	< 2	< 2	< 2	16	4096	< 2	< 2		
33	< 2	< 2	< 2	< 2	4096	4096	4096	ND	
34	< 2	< 2	< 2	< 2	< 2	4096	4096	4096	< 2

ND = Not Done.

* Results are expressed as the reciprocal of the highest titre giving complete haemagglutination.

TABLE 38. CHAPTER 7 : EXAMINATION OF INTESTINAL CONTENTS FOR THE PRESENCE OF SPECIFIC CPV HAEMAGGLUTININ*

Dog No.	0	1	2	3	4	5	6	7	8
25	IL < 2								
26	IL < 2								
27		IL < 2							
28			IL < 2						
			Co < 2						
29				IL < 2					
30					IL 4096				
					St < 2				
31						IL 4096			
						St 4096			
32							IL < 2		
							St < 2		
33								IL < 2	
								St < 2	
								Co < 2	
34									IL < 2
									St < 2

IL = Ileum contents. St = Stomach contents. Co = Colon.

* Results are expressed as the reciprocals of the highest dilution giving complete agglutination

TABLE 39.

CHAPTER 7 : EXAMINATION OF SERA FOR THE PRESENCE
OF HAEMAGGLUTINATION INHIBITING ANTIBODY TO CPV*

Dog No.	Day Post Infection							
	0	1	2	3	4	5	6	7
25	<4							
26	<4							
27	<4	<4						
28	<4	<4	<4					
29	<4	<4	<4	<4				
30	<4	<4	<4	<4	<4			
31	<4	<4	<4	<4	<4	8		
32	<4	<4	<4	<4	<4	16	256	
33	<4	<4	<4	<4	<4	64	256	4096
34	<4	<4	<4	<4	<4	16	128	2048

* Results are expressed as the reciprocal of the highest dilution which completely inhibited haemagglutination.

TABLE 40.

CHAPTER 7 : EXAMINATION OF FAECAL SPECIMENS FOR THE PRESENCE OF
HAEMAGGLUTINATION-INHIBITING ANTIBODY TO CPV*

Dog No.	Day Post Infection							
	0	1	2	3	4	5	6	7
25	<4							
26	<4							
27	<4	<4						
28	<4	<4	<4					
29	<4	<4	<4	<4				
30	<4	<4	<4	<4	<4			
31	<4	<4	<4	<4	<4	<4		
32	<4	<4	<4	<4	<4	<4	<4	
33	<4	<4	<4	<4	<4	<4	<4	<4
34	<4	<4	<4	<4	<4	<4	<4	32

* Results are expressed as the reciprocal of the highest dilution which completely inhibited haemagglutination.

TABLE 41. CHAPTER 7 : EXAMINATION OF INTESTINAL AND GASTRIC CONTENTS FOR THE PRESENCE OF HAEMAGGLUTINATION-INHIBITING ANTIBODY TO CPV.*

Dog No.	0	1	2	3	4	5	6	7	8
25	IL < 4								
26	IL < 4								
27		IL < 4							
28			IL < 4						
29				IL < 4					
30					St < 4				
					ND				
31						ND			
32							IL < 4		
							St < 4		
33								IL 32	
								St < 4	
								Caecum 4	
									IL 256
34									St < 4

ND = Not Done. IL = Ileum contents. St = Stomach contents.

* Results are expressed as the reciprocal of the highest dilution which completely inhibits haemagglutination.

TABLE 42.

CHAPTER 7: POST MORTEM FINDINGS

Dog No.	Day Killed	Macroscopic Findings
25	0	NAD
26	0	NAD
27	1	NAD
28	2	NAD
29	3	Slight thymic oedema.
30	4	Thymic oedema and petechiae.
31	5	Slight reduction in thymic size, enlargement and oedema of lymph nodes.
32	6	Small thymus, enlargement of lymph nodes, except mesenteric. Wall of duodenum thickened and haemorrhagic.
33	7	Small thymus, lymph nodes enlarged, except mesenteric. Wall of duodenum and jejunum thickened.
34	8	Small thymus, lymph nodes enlarged.

TABLE 43.

CHAPTER 7: QUANTIFICATION OF TOTAL BODY WEIGHT AND THYMIC WEIGHT TOGETHER WITH THE BODY WEIGHT TO THYMIC WEIGHT RATIO.

Dog No.	Day Killed	Body Wt. (g)	Thymic Wt. (g)	<u>Body Wt.</u> <u>Thymic Wt.</u>
25	0	3100	14	221
26	0	3865	15	257
27	1	5615	25	224
28	2	4115	21	195
29	3	2945	11	267
30*	4	3994	23	173
31	5	3450	9.5	363
32	6	7350	8	543
33	7	3125	2.25	1388
34	8	4540	5.5	825

* Increase in thymic mass in this animal apparently the result of marked thymic oedema.

TABLE 44.

CHAPTER 7: MICROSCOPIC LYMPHOID FINDINGS

Dog No.	Day Killed	Microscopic Lymphoid Findings
25	0	NAD
26	0	NAD
27	1	NAD
28	2	Pyknosis and fragmentation lymphocytes in thymic cortex and germinal centres.
29	3	Widespread lymphocytolysis in thymic cortex and nodes.
30	4	Widespread lymphocytolysis in thymic cortex and nodes. Interlobular thymic oedema.
31	5	Depletion thymic cortex and germinal centres.
32	6	Extensive depletion of thymic cortex and germinal centres.
33	7	Thymic cortex depleted. Repopulation of germinal centres.
34	8	Thymic cortex depleted. Repopulation and expansion germinal centres. Some expansion of paracortex of nodes.

TABLE 45.

CHAPTER 7: CLASSIFICATION OF INTESTINAL LESIONS.

Dog No.	Day Killed	Duodenum		Jejunum		Ileum	
		Pattern	Confluency	Pattern	Confluency	Pattern	Confluency
25	0	-		-		-	
26	0	-		-		-	
27	1	-		-		-	
28	2	-		-		-	
29	3	-		-		-	
30	4	A	++	A	+	-	
31	5	A	++	A	+	A	+++
32	6	B	+++	B	+	A	+++
33	7	D	++	B/D	++	A/D	+
34	8	D	++	D	++	D	++

* Mucosa overlying GALT only.

TABLE 46

CHAPTER 7: DISTRIBUTION OF VIRAL ANTIGEN BY IMMUNOCYTOCHEMISTRY.

Dog No.	Day Killed	Thymus	Lymph Node	Spleen	Duodenum	Jejunum	Ileum	Colon	Bone Marrow
25	0								
26	0								
27	1	+	-	-	-	-	-	-	-
28	2	++	+	-	-	-	-	-	-
29	3	+++	++	+	-	-	-	-	-
30	4	++	++	+	+	+	+	-	-
31	5	+	+	+	+++	+	+	-	+
32	6	+	++	+	++	+++	+	+	+
33	7	±	+	+	+	+	++	++	-
34	8	-	±	-	-	-	-	-	-

Fig. 52: Canine Parvovirus Infection - Thymus of Dog 27

Individual positively stained thymocytes are present.
These cells were seen throughout the thymic cortex.
Taken at 1 DPI.

Immunofluorescent stain x 400.

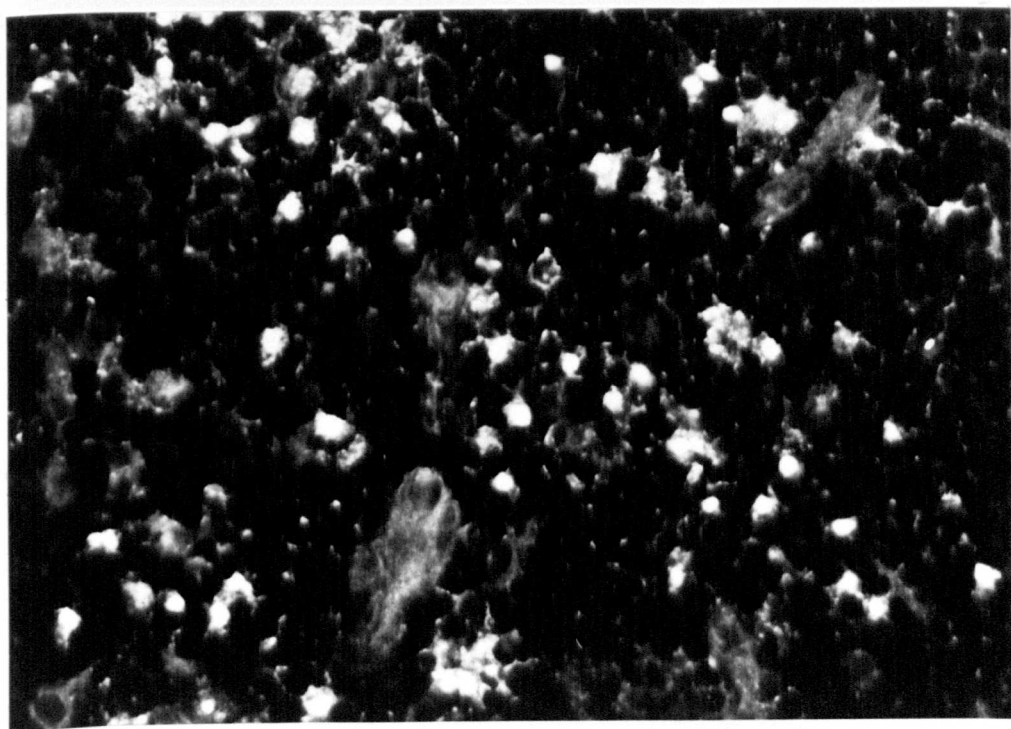


Fig. 53: Canine Parvovirus Infection - Lymph Node of Dog 29.

There is bright fluorescence in the germinal centre.
Taken at 3 DPI.

Immunofluorescent stain x 250.

Fig. 54: Canine Parvovirus Infection - Lymph Node of Dog 29.

Note the dark brown staining in the germinal centre.
Antigen is predominantly intranuclear. Taken at 3
DPI.

Immunoperoxidase stain x 100.

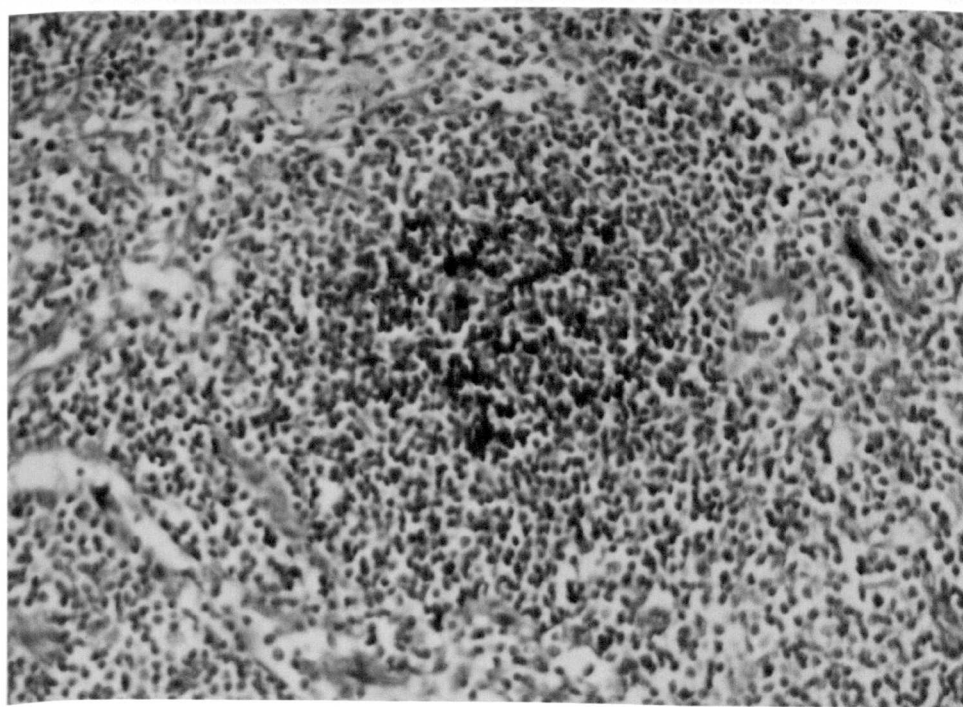
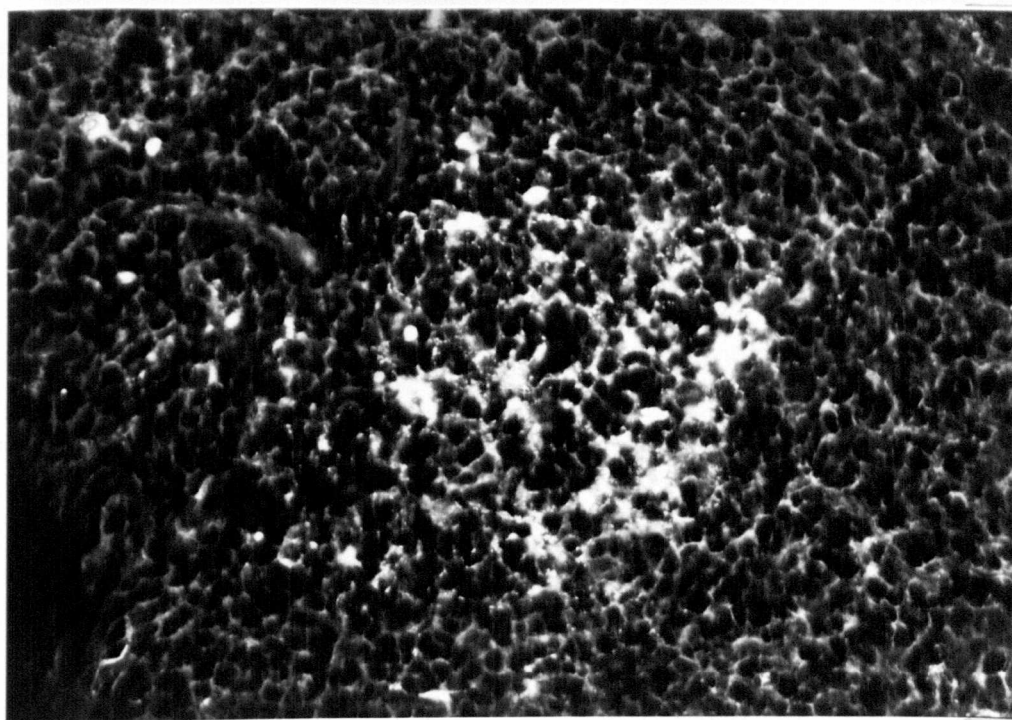


Fig. 55: Canine Parvovirus Infection - Thymus of Dog 30.

There is extensive fluorescence of individual cells in the cortex of the thymus. Less dense autofluorescence may be seen in the interlobular connective tissue and in the reticuloepithelial cells of the medulla.

Taken at 4 DPI.

Immunofluorescent stain x 100.

Fig. 56: Canine Parvovirus Infection - Thymus of Dog 30.

Specific staining is predominantly intranuclear. However small amounts of antigen may be seen in the intercellular connective tissue (arrow). Taken at 4 DPI.

Immunoperoxidase stain x 250.

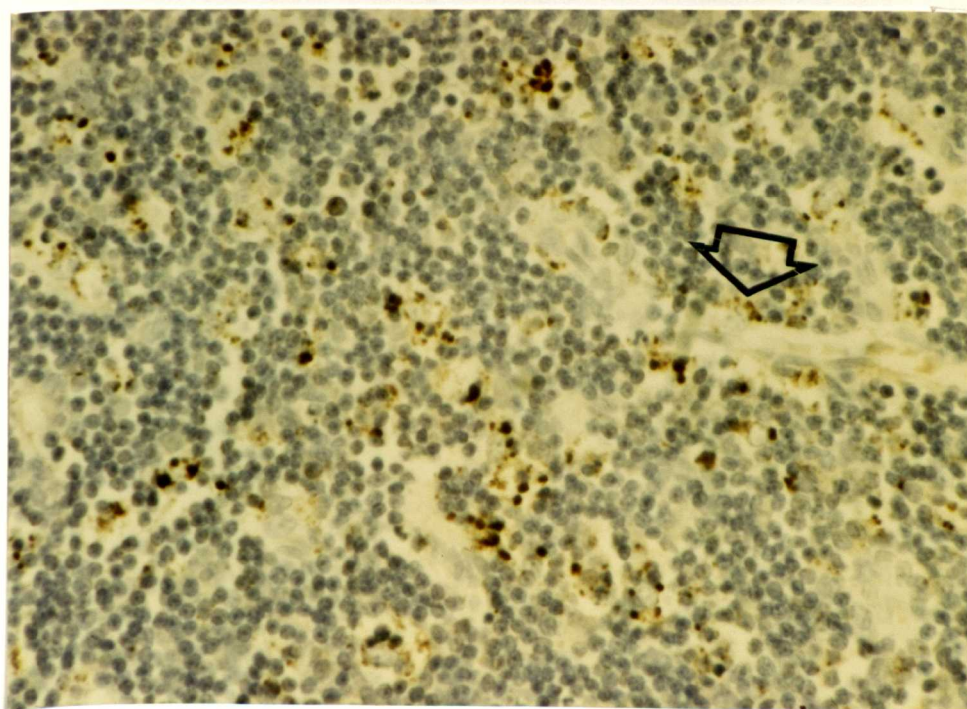
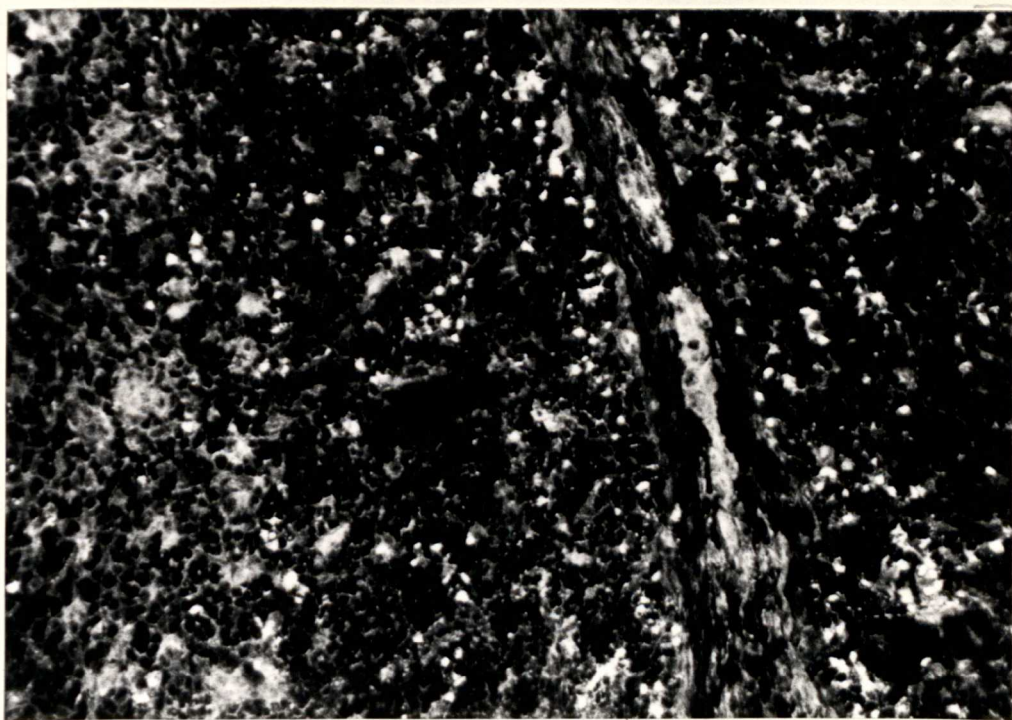


Fig. 57: Canine Parvovirus Infection - Intestine of Dog 30.

Single cells in the crypt epithelium are positively stained. These cells are restricted to the proliferative zone, with no staining in the basal crypt cells. Taken at 4 DPI.

Immunofluorescent stain x 250.

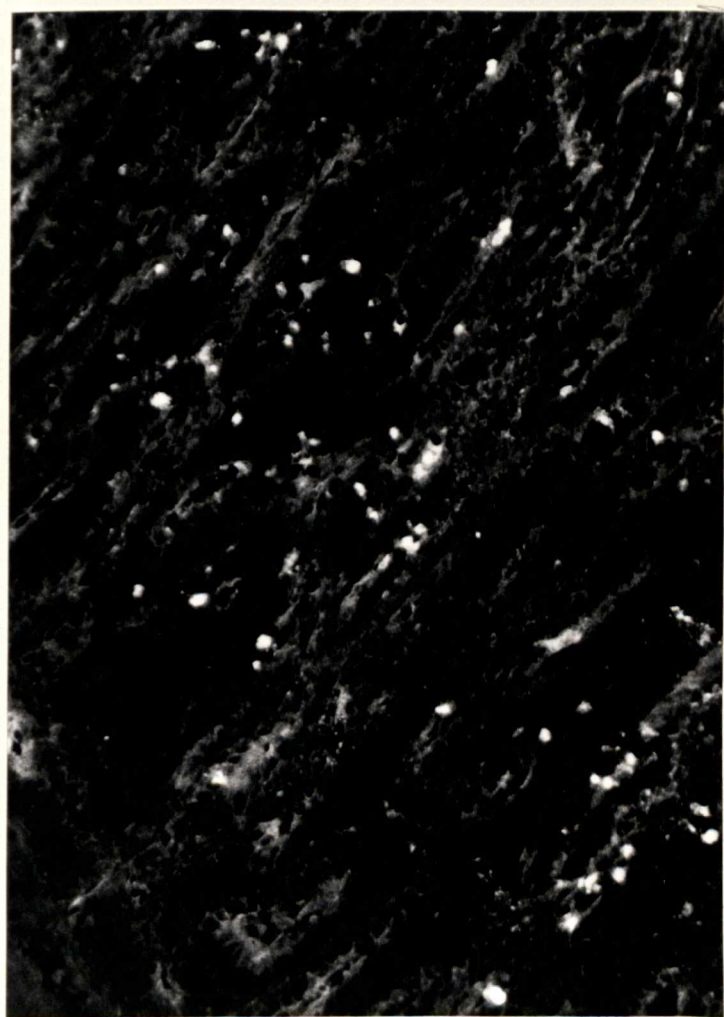


Fig. 58: Canine Parvovirus Infection - Intestine of Dog 30.

Small darkly staining cells may be seen in the crypt epithelium. The position and morphology of the cells correspond to the dark amphiphilic cells seen on conventional histological examination. Taken at 4 DPI.

Immunoperoxidase stain x 250.

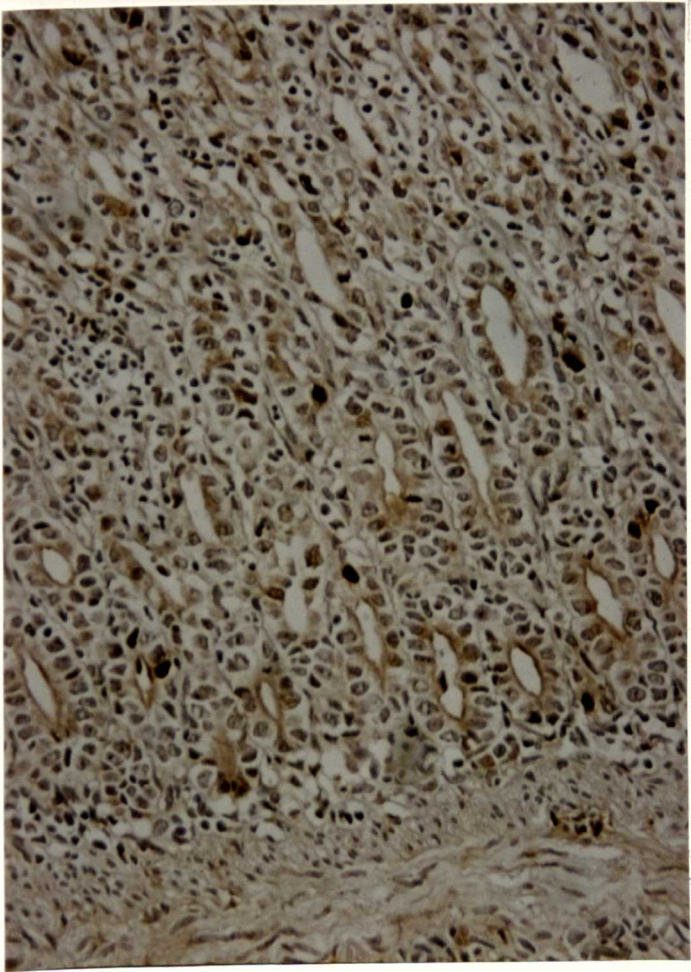


Fig. 59: Canine Parvovirus Infection - Intestine of Dog 30.

There is specific fluorescence in crypt enterocytes.
Taken at 4 DPI.

Immunofluorescent stain x 400.

Fig. 60: Canine Parvovirus Infection - Intestine of Dog 30.

Single darkly staining cells may be seen in the epithelium lining the crypt. These cells correspond in size and morphology to the small darkly amphophilic staining cells seen on conventional light microscopy.
Taken at 4 DPI.

Immunoperoxidase stain x 400.

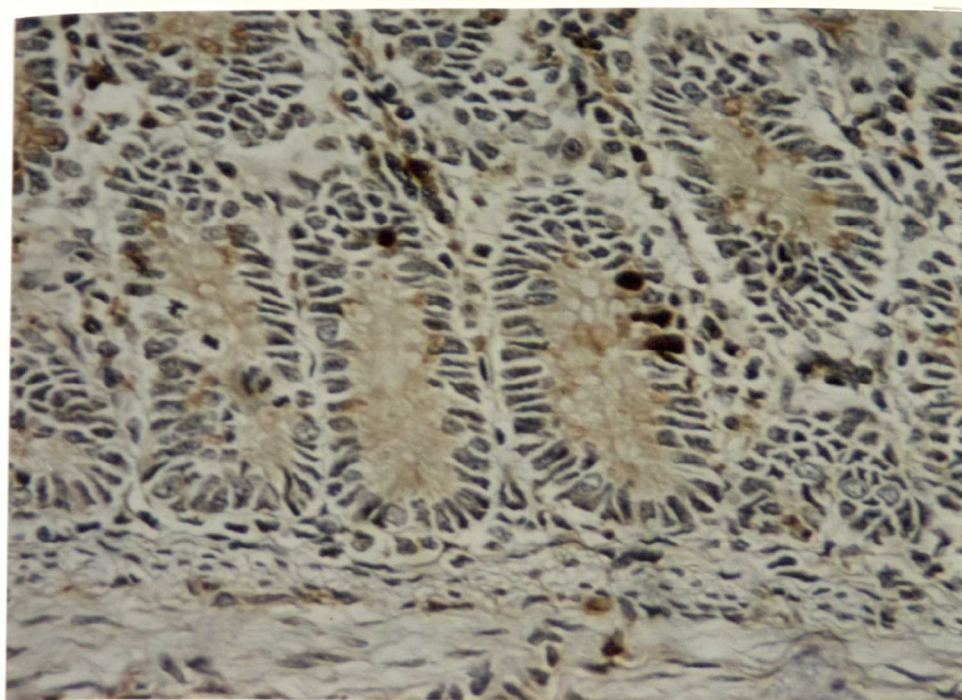
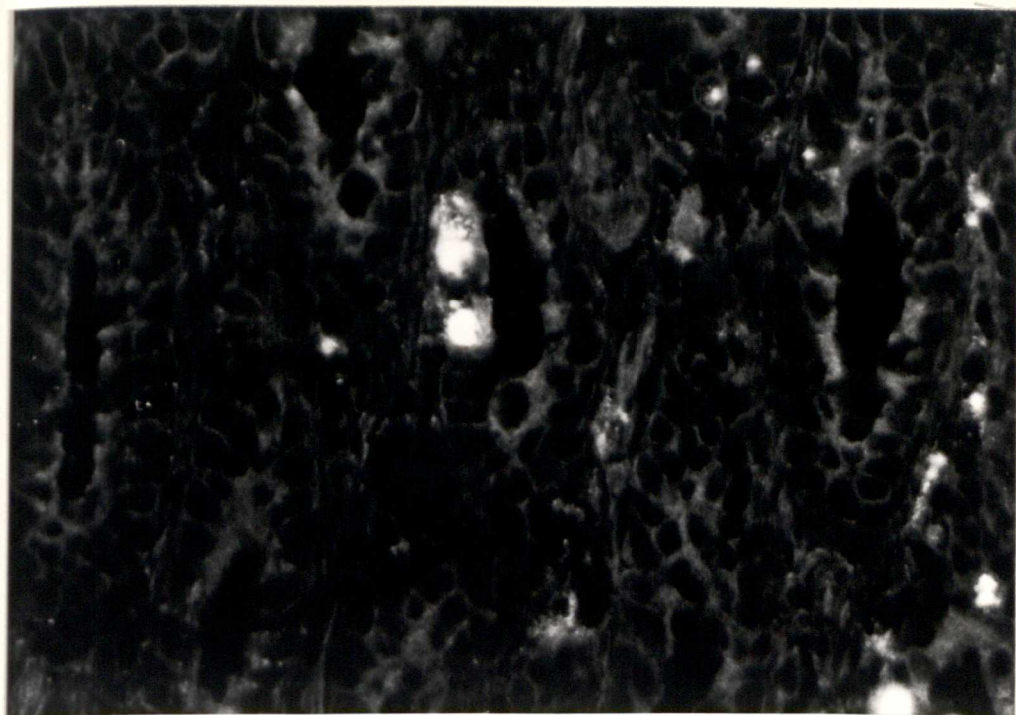


Fig. 61: Canine Parvovirus Infection - Lymph Node of Dog 31

There is specific intranuclear staining of lymphocytes in the germinal centre. Antigen is also apparent in the intercellular spaces. Taken at 5 DPI.

Immunofluorescent stain x 250.

Fig. 62: Canine Parvovirus Infection - Lymph Node of Dog 31

Specific staining is both intranuclear and in intercellular spaces. Taken at 5 DPI.

Immunoperoxidase stain x 250.

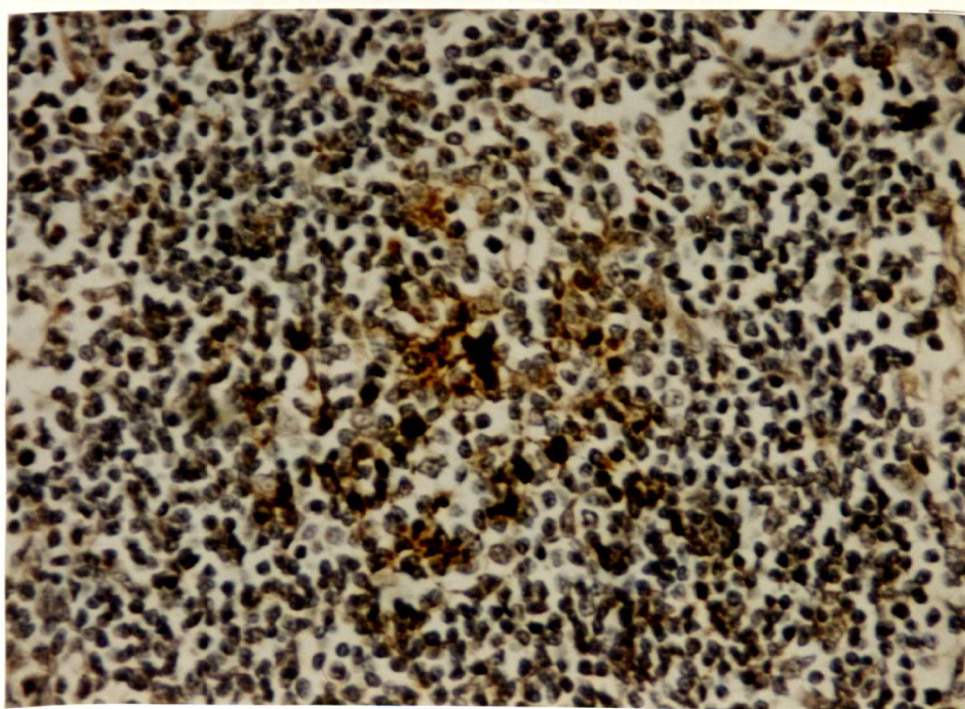
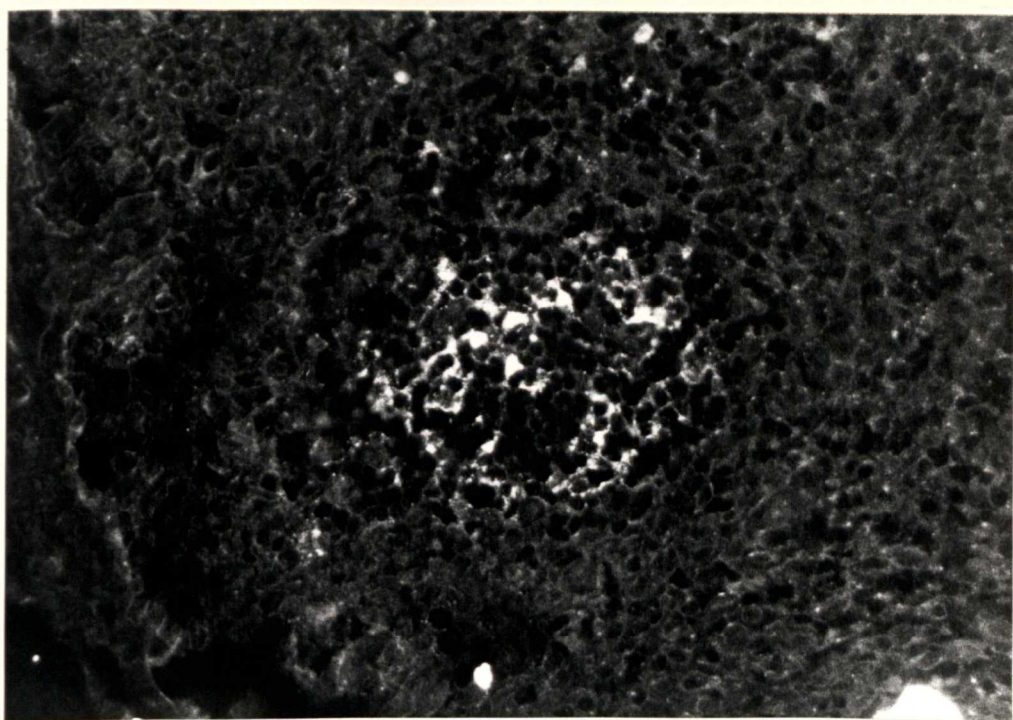


Fig. 63: Canine Parvovirus Infection - Liver of Dog 31.

Specific staining may be observed in the cytoplasm of Von Kupffer cells. Taken at 5 DPI.

Immunofluorescent stain x 250.

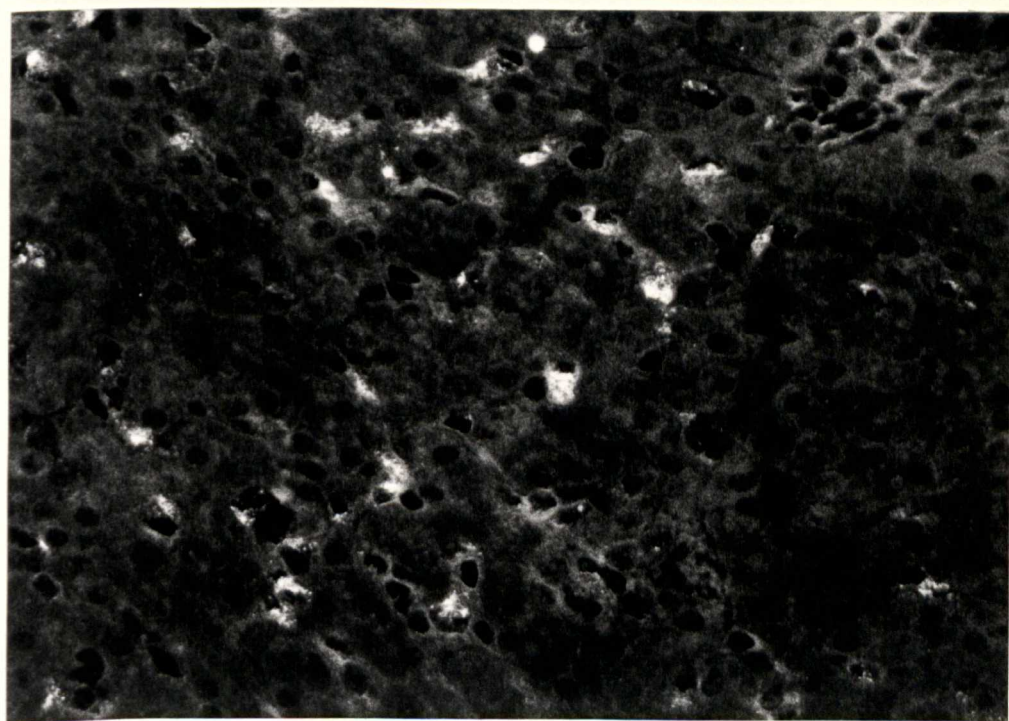


Fig. 64: Canine Parvovirus Infection - Lymph Node of Dog 32.

Antigen is present both in the nucleus of lymphocytes and in the extracellular spaces. Staining is confined to the lymphoid corona at the periphery of the germinal centre, there is no antigen in the histiocytic reticulum cells in the germinal centre itself. Taken at 6 DPI.

Immunoperoxidase stain x 100.

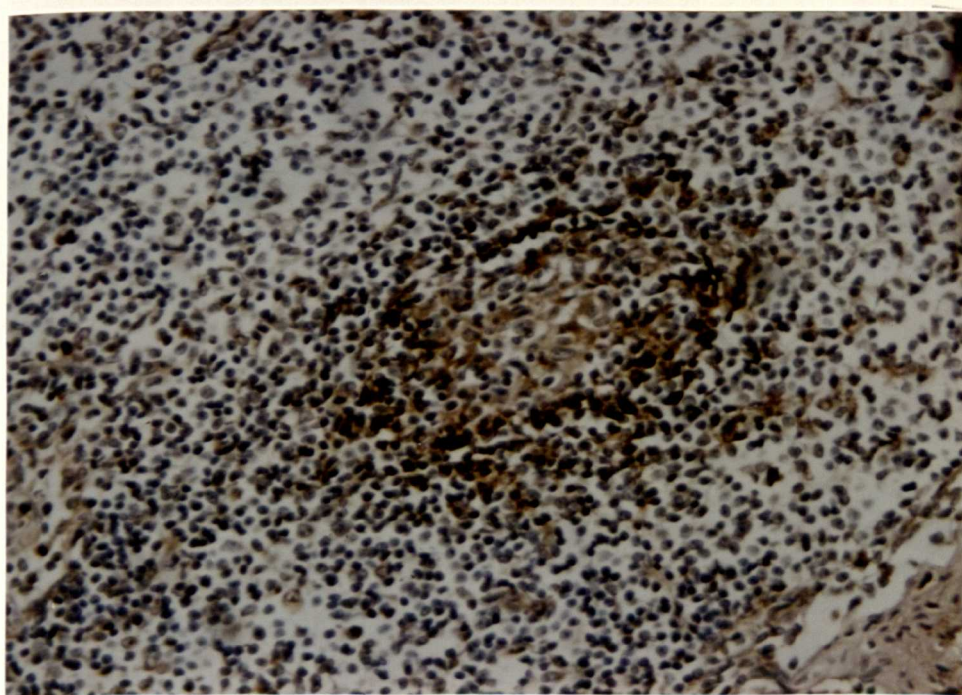


Fig. 65: Canine Parvovirus Infection - Marrow of Dog 32.

There is specific fluorescence in medium-sized cells, with megakaryocytes unaffected. Taken at 6 DPI.

Immunofluorescent stain x 400.

Fig. 66: Canine Parvovirus Infection - Marrow of Dog 32.

There is dense positive staining in the nuclei of cells in the bone marrow, however identification of the cells is not possible since the stain obscures nuclear morphology. Taken at 6 DPI.

Immunoperoxidase stain x 400.

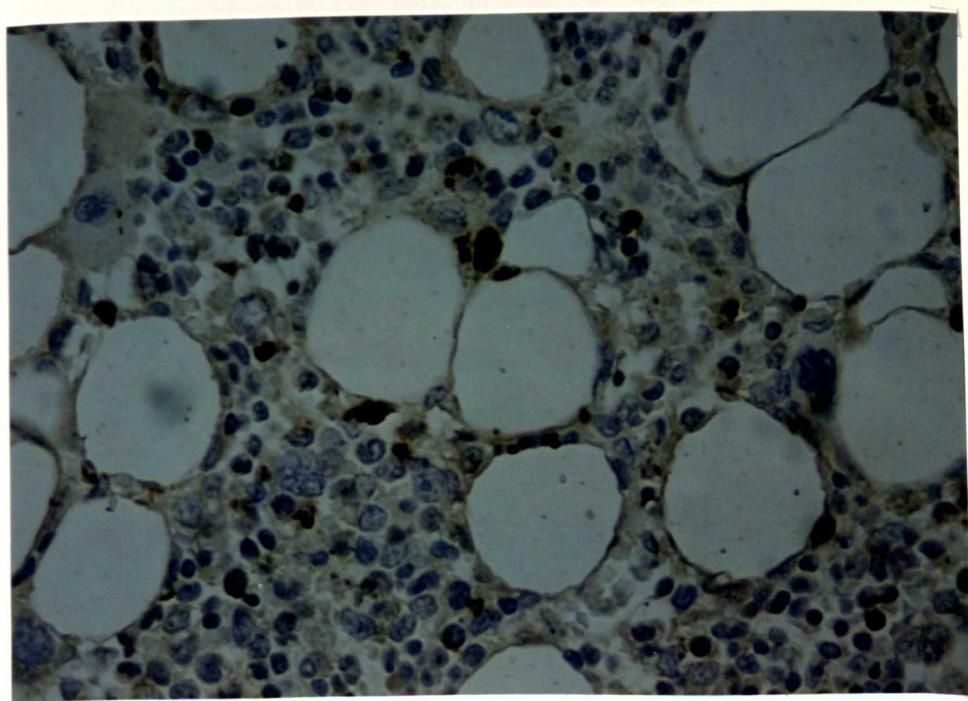
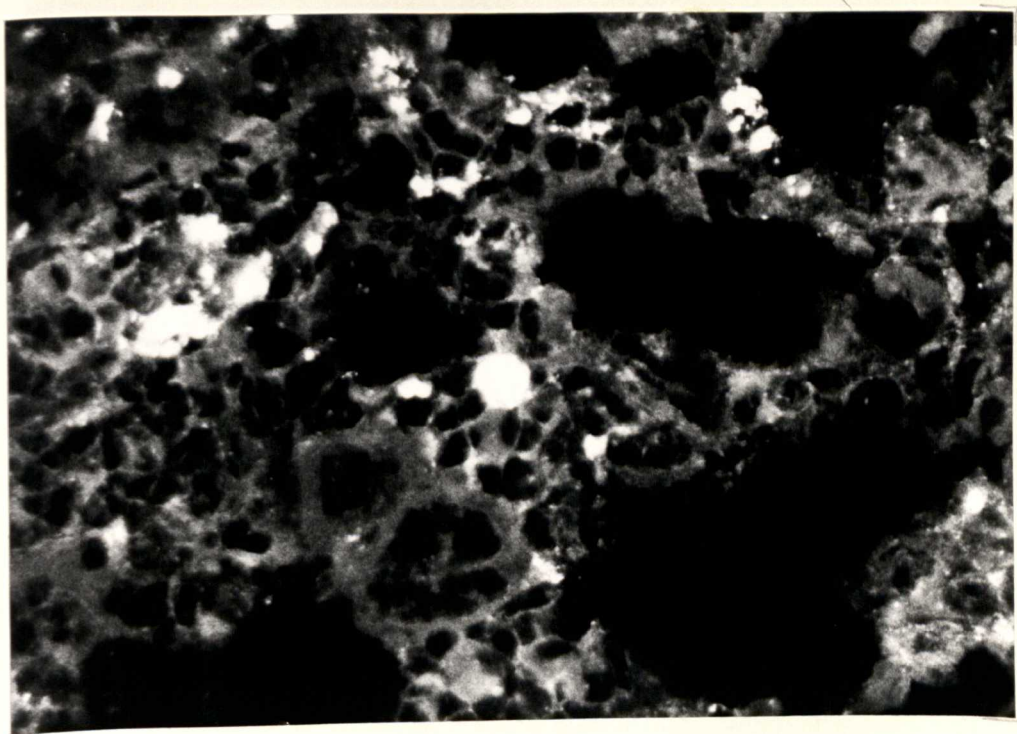


Fig. 67: Canine Parvovirus Infection - Intestine of Dog 32.

Specific staining may be observed in cells located in the crypt epithelium. However antigen is also present in the lumen of the crypts, although here it appears more granular. Taken at 6 DPI.

Immunofluorescent stain x 400.

Fig. 68: Canine Parvovirus Infection - Intestine of Dog 32.

At this low power the distribution of antigen in the mucosa may be observed. Specific staining is localised to the proliferative zone of the crypt epithelium and is present both in epithelial cells and, moreover, within the lumina of the crypts. Taken at 6 DPI.

Immunoperoxidase stain x 40.

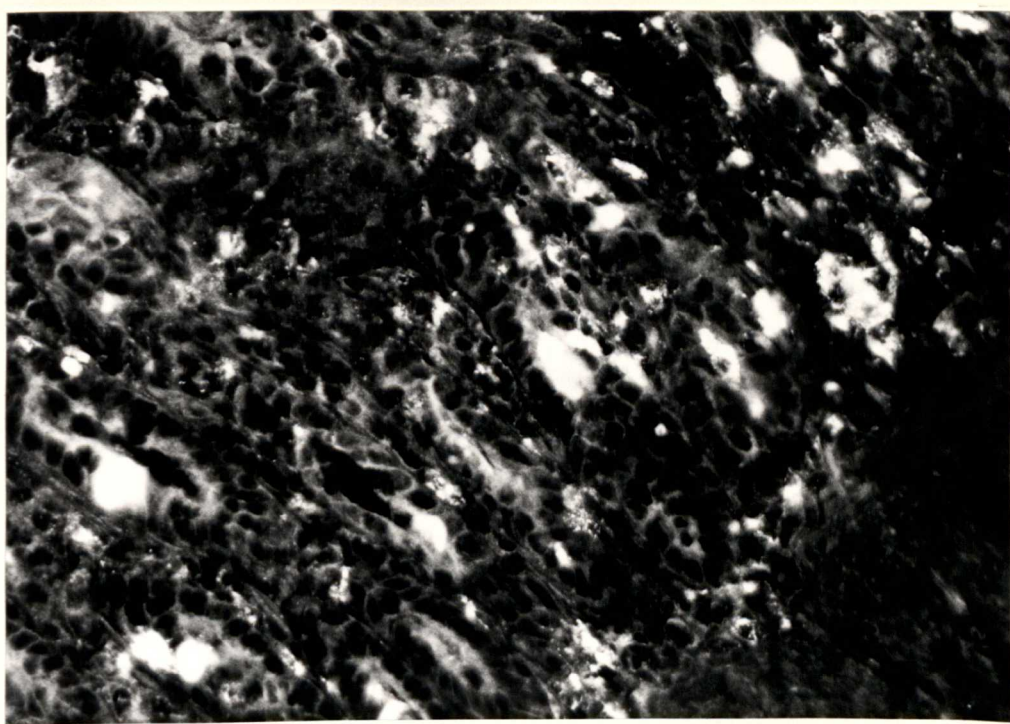


Fig. 69: Canine Parvovirus Infection - Lymph Node of Dog 33.

There is specific fluorescence in the germinal centre, however, antigen does not appear to be intranuclear but is instead in the extracellular spaces. Taken at 7 DPI.

Immunofluorescent stain x 250.

Fig. 70: Canine Parvovirus Infection - Lymph Node of Dog 33.

Small amounts of specific staining may be observed outwith the lymphocytes of the germinal centre. This distribution of antigen suggests it is extracellular. However, it is possible that it may be within the cytoplasm of histiocytic reticulum cells, although using only a nuclear counter stain, this is difficult to determine accurately.

Immunoperoxidase stain x 400.

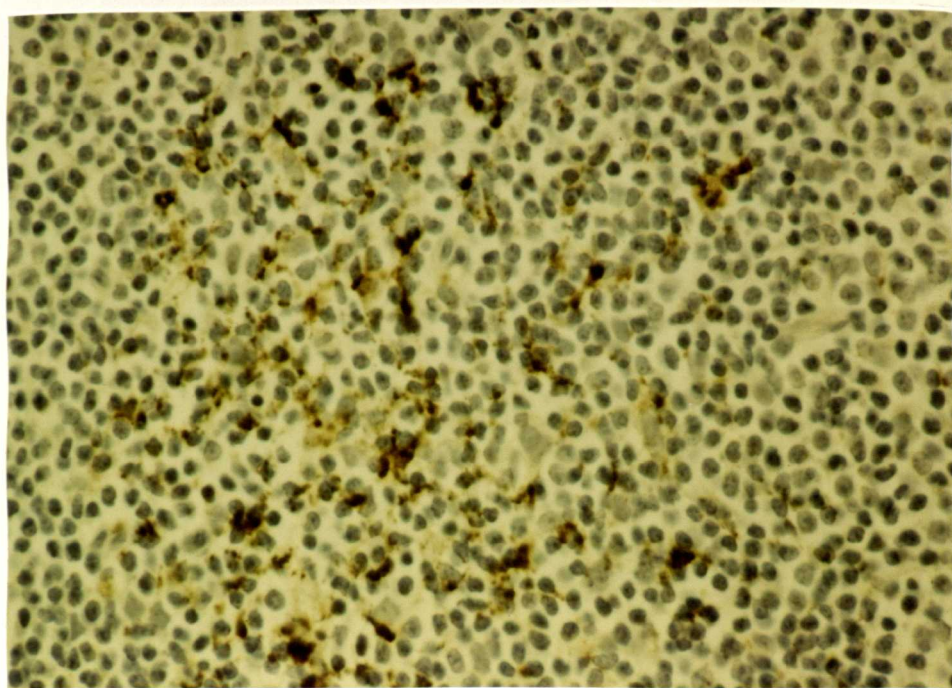
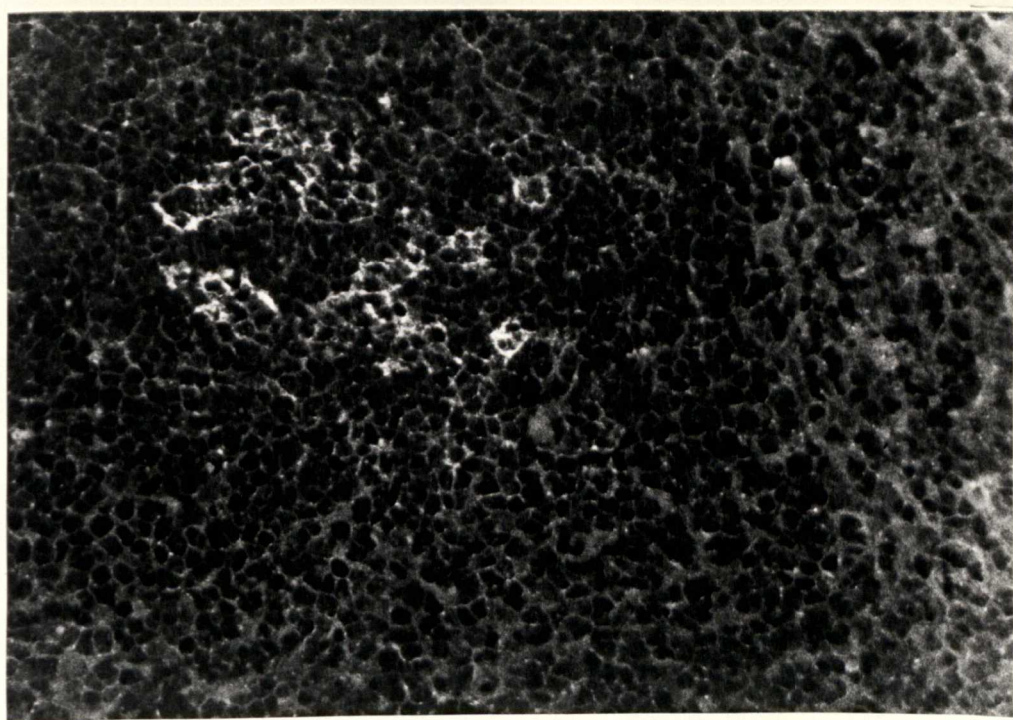


Fig. 71: Duodenum of Control Dog.

The villi are finger-like in shape and are of a uniform height.

x 480.

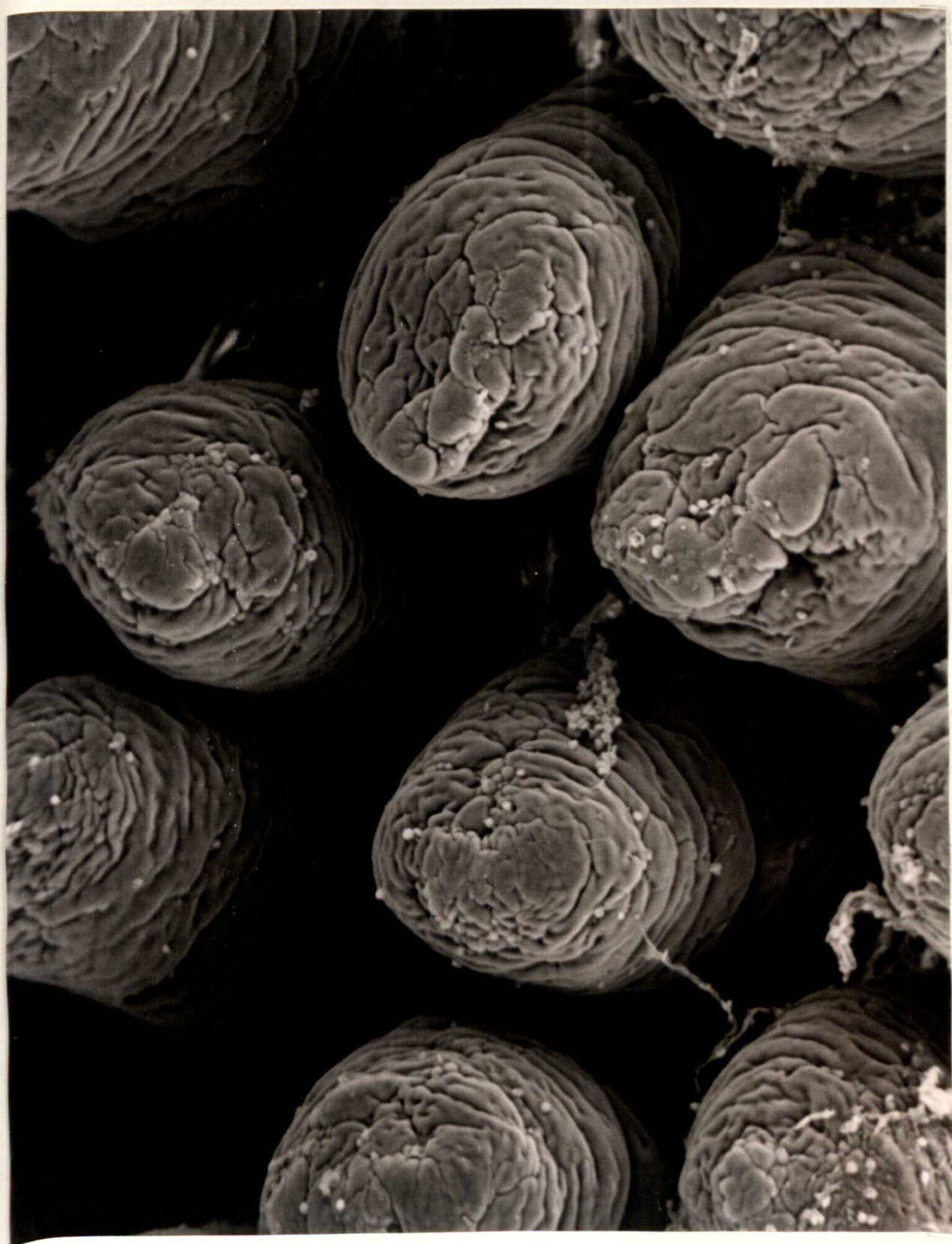


Fig. 72: Ileum of Control Dog.

The villi are again finger-like in shape and are of uniform height. However, they appear slightly more slender than those in the duodenum. Numerous shallow irregular grooves may be seen on the surface of the villi.

x 480

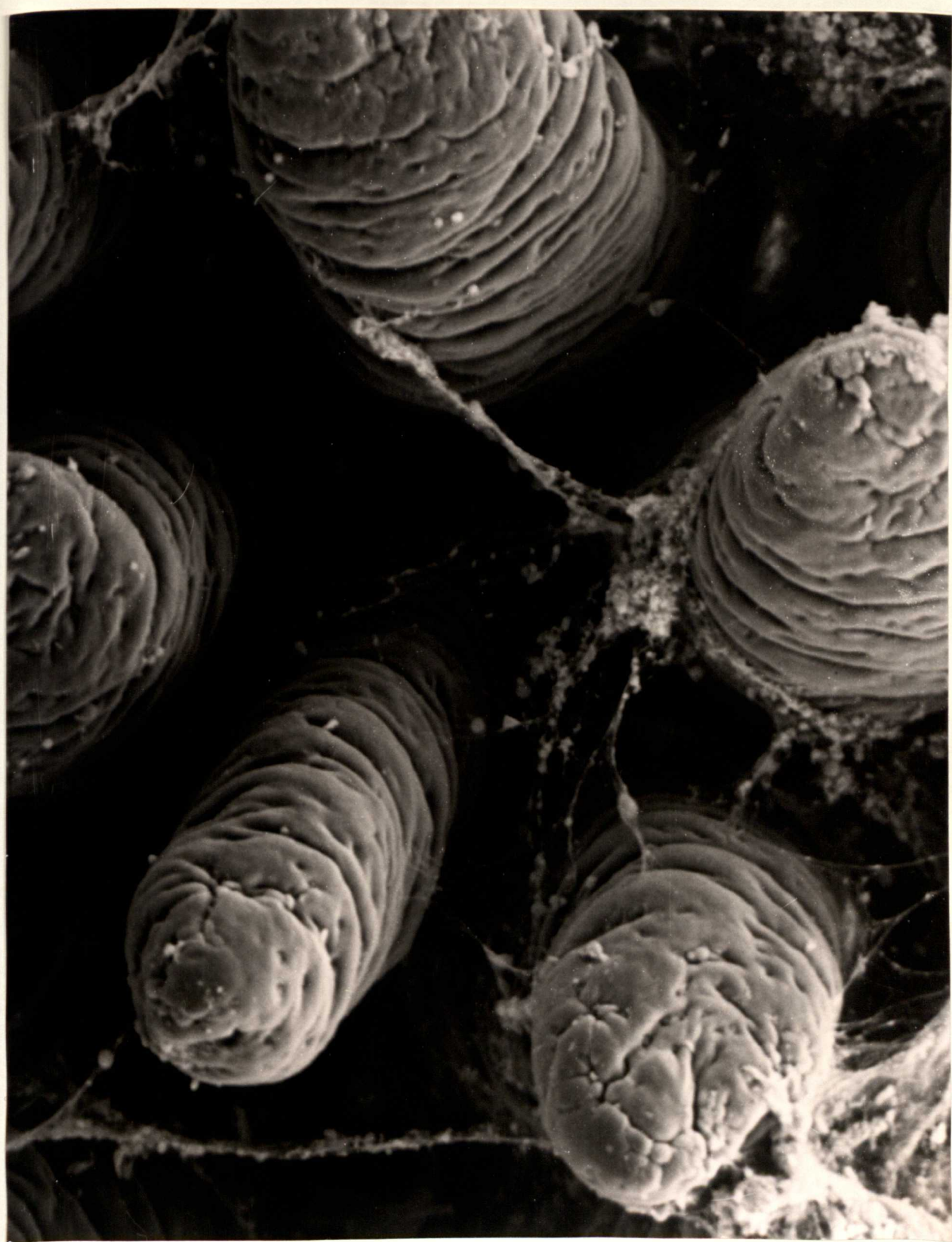


Fig. 73: Duodenum of Control Dog.

The epithelial cells are covered by a velvety pile of microvilli. However, the outlines of individual cells may be discerned as slightly raised ridges on the epithelial surface (arrow). Cells at the apical extrusion zone are separated, rounded and have lost surface microvilli.

x 1920.



Fig. 74 Ileum of Control Dog.

There are numerous pits in the epithelium representing the openings of goblet cells.

x 960.

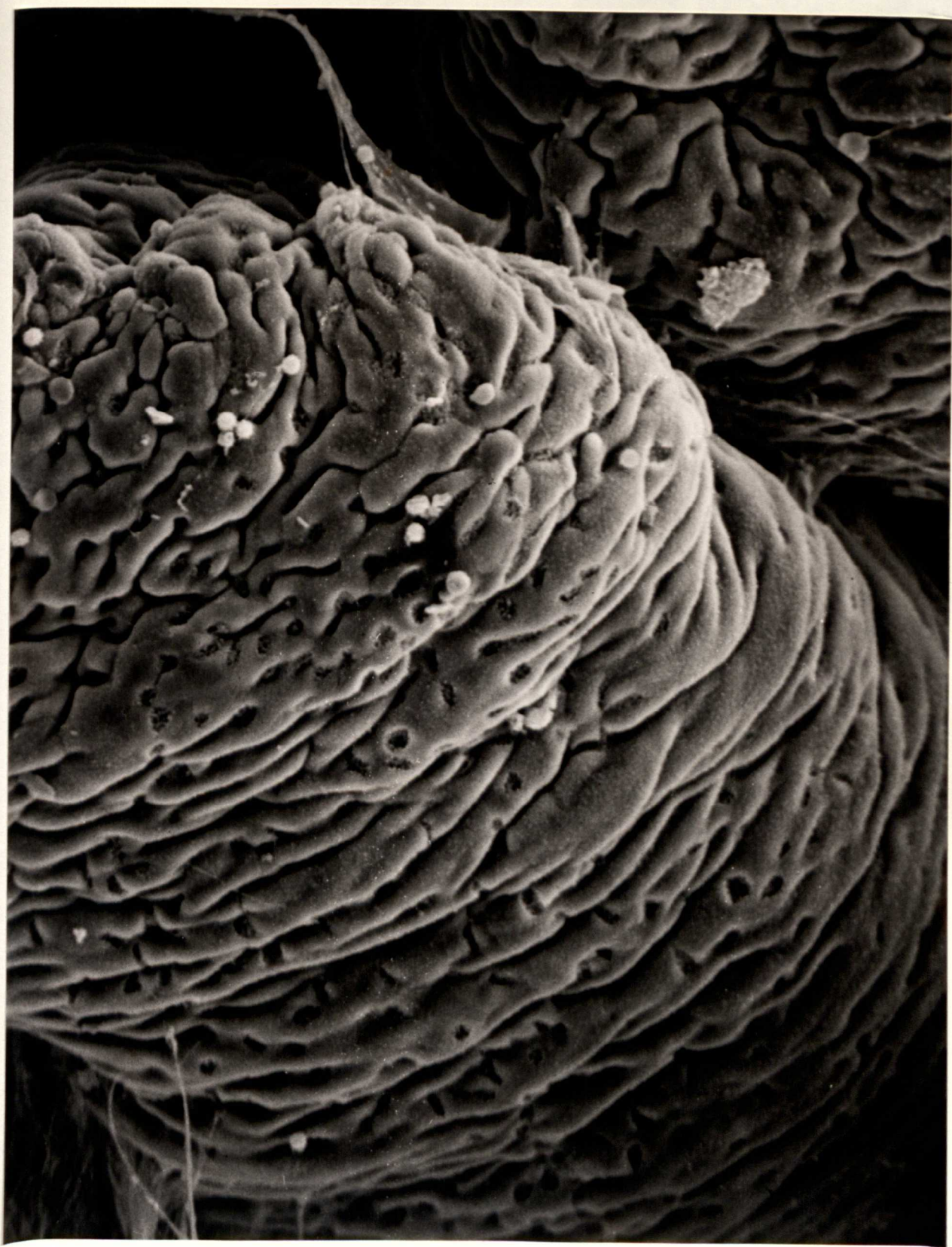


Fig. 75: Jejunum of Control Dog.

The circumvillar basin is visible in this specimen and numerous crypt openings may be seen.

x 480.

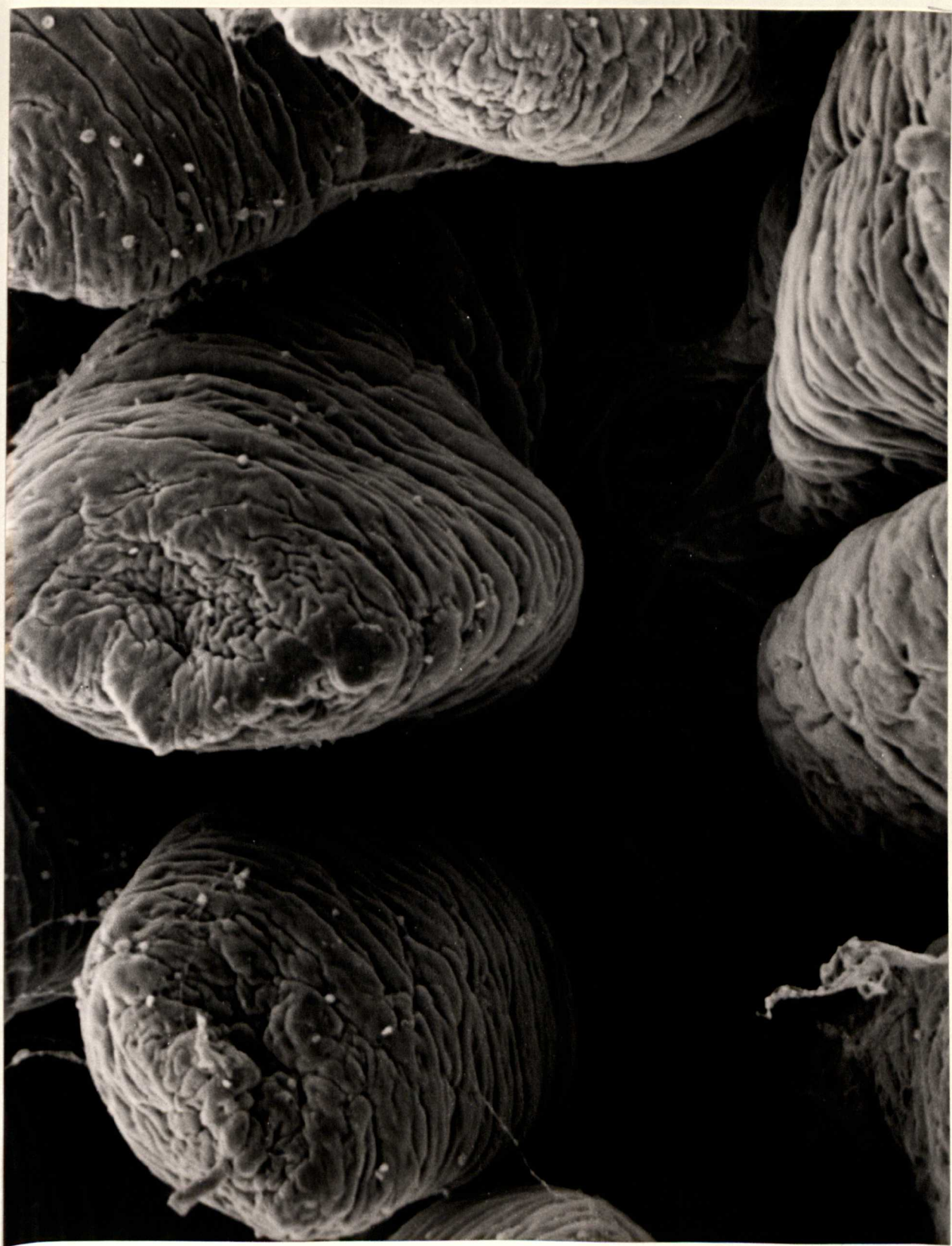


Fig. 76: Colon of Control Dog.

Crypt openings are regularly arranged and some contain mucus and cellular debris. There are no villi.

x 480.



Fig. 77: Canine Parvovirus Infection - Duodenum of Dog 32.

Villi are shortened and may be discerned only through breaks in the thick sheet of mucus, the edge of which is arrowed. Mucus covered most of the surface of the small intestine. Taken at 6 DPI.

x 480.

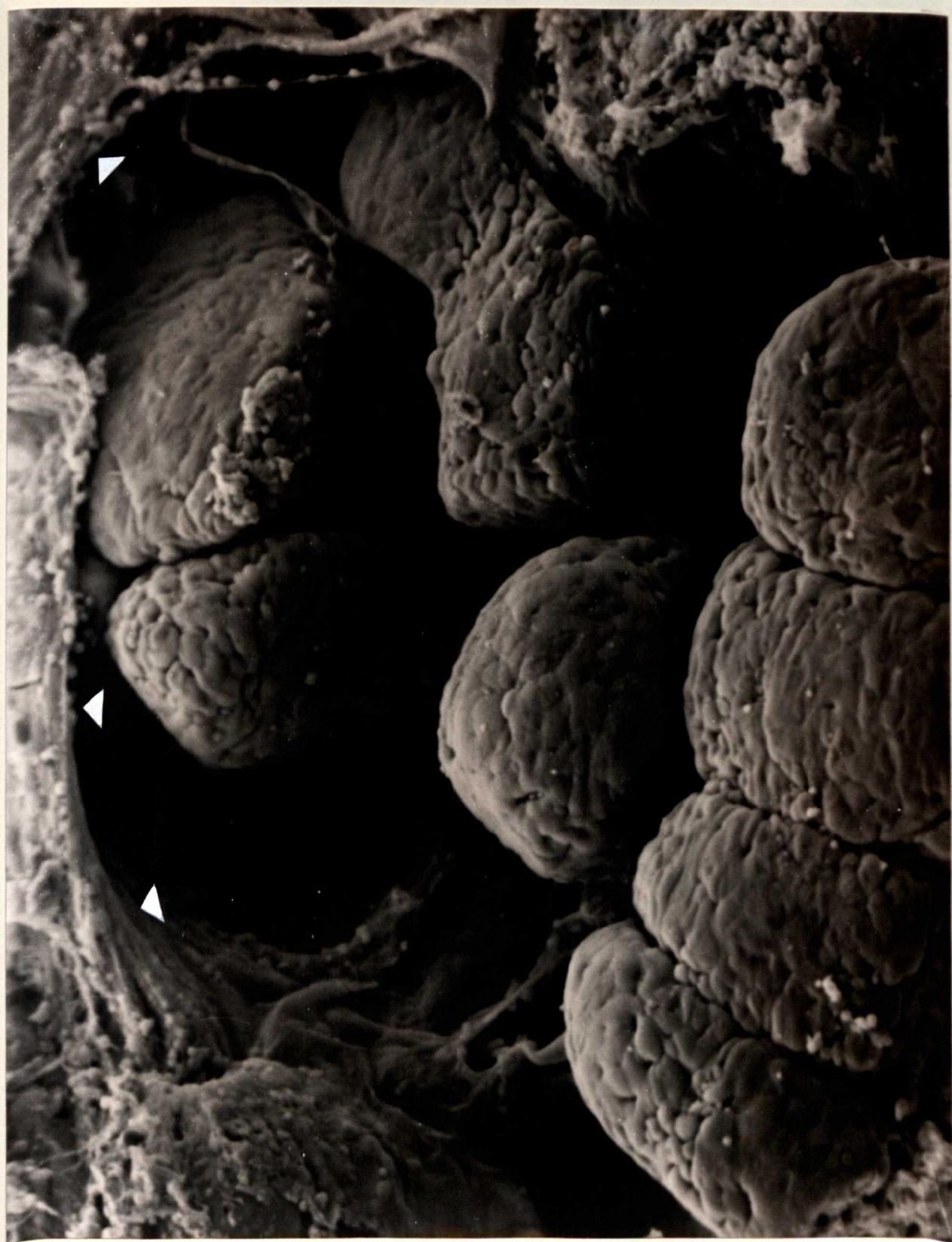


Fig. 78: Canine Parvovirus Infection - Duodenum of Dog 32.

The villi are shortened, irregular in height, and in some cases are fused to adjacent villi. The circumvillar basin at the base of the villi and the crypt openings are dilated. Taken at 6 NPI.

x 480.

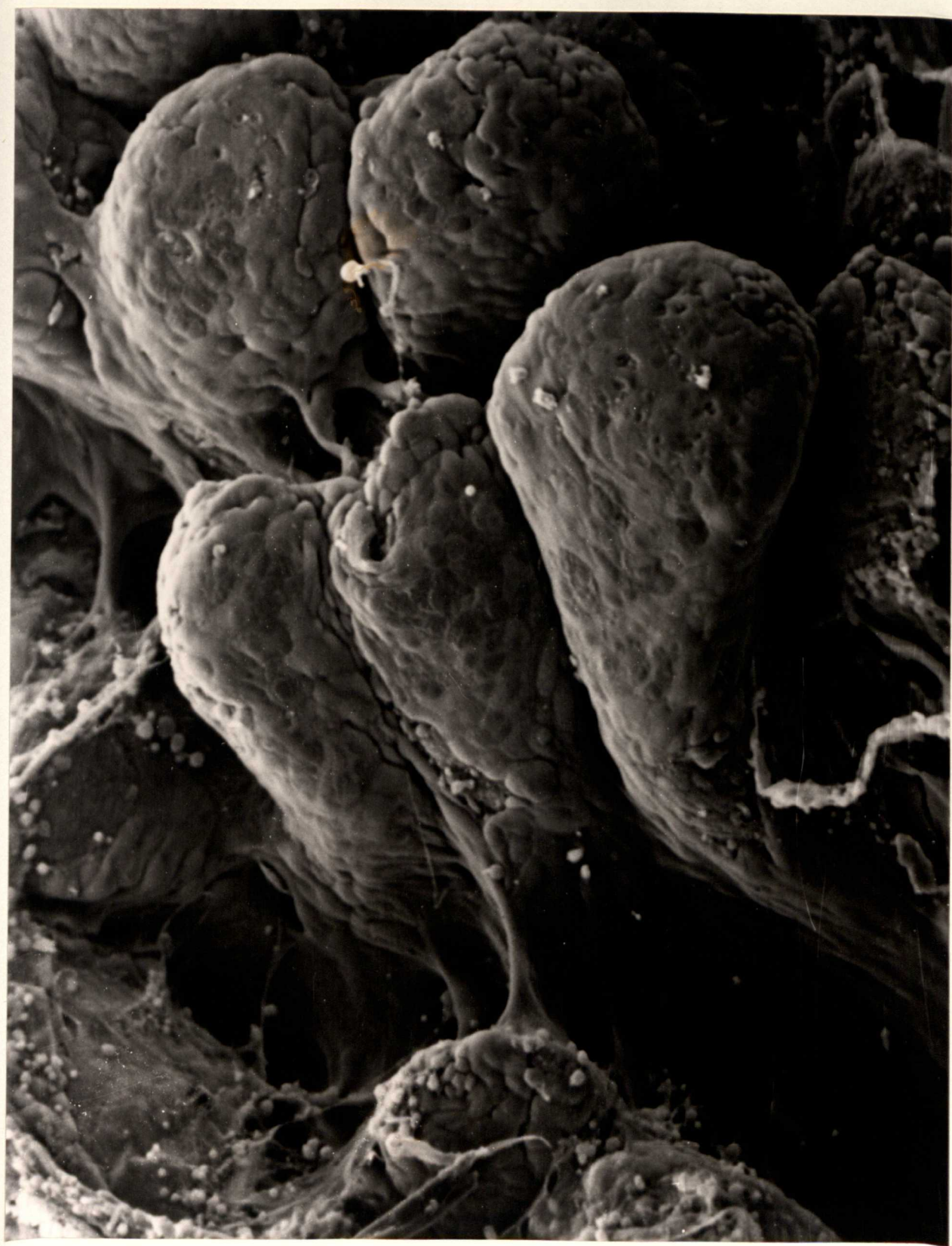


Fig. 79: Canine Parvovirus Infection - Duodenum of Dog 32.

The epithelial cells at the tips of the villi are absent and the underlying lamina propria exposed
Taken at 6 DPI.

x 960.



Fig. 80: Canine Parvovirus Infection - Jejunum of Dog 32.

The villi are reduced to stubby projections on the surface of the mucosa. In some areas the surface epithelium has been lost although some villi at the edge of these severely affected areas remain partially clothed in epithelium on some surfaces. Taken at 6 DPI.

x 480.



Fig. 81: Canine Parvovirus Infection - Ileum of Dog 32.

Villi are generally shortened, and some leaf-shaped villi may be seen. There are large numbers of discharged goblet cells present in the epithelium. Taken at 6 DPI.

x 480.

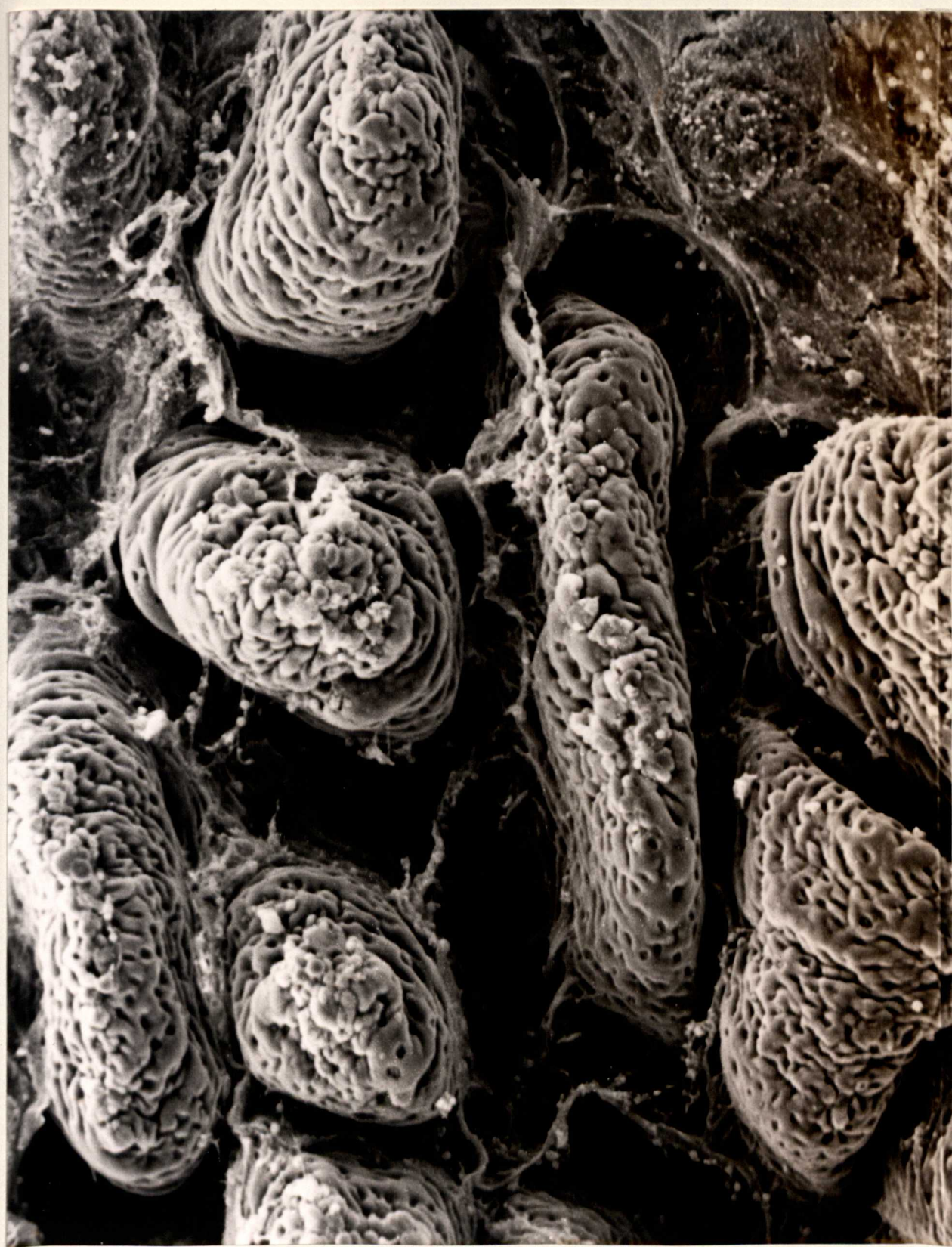


Fig. 82: Canine Parvovirus Infection - Jejunum of Dog 33.

Villi may be discerned as short conical protuberances on the surface of the mucosa. Taken at 7 DPI.

x 480.

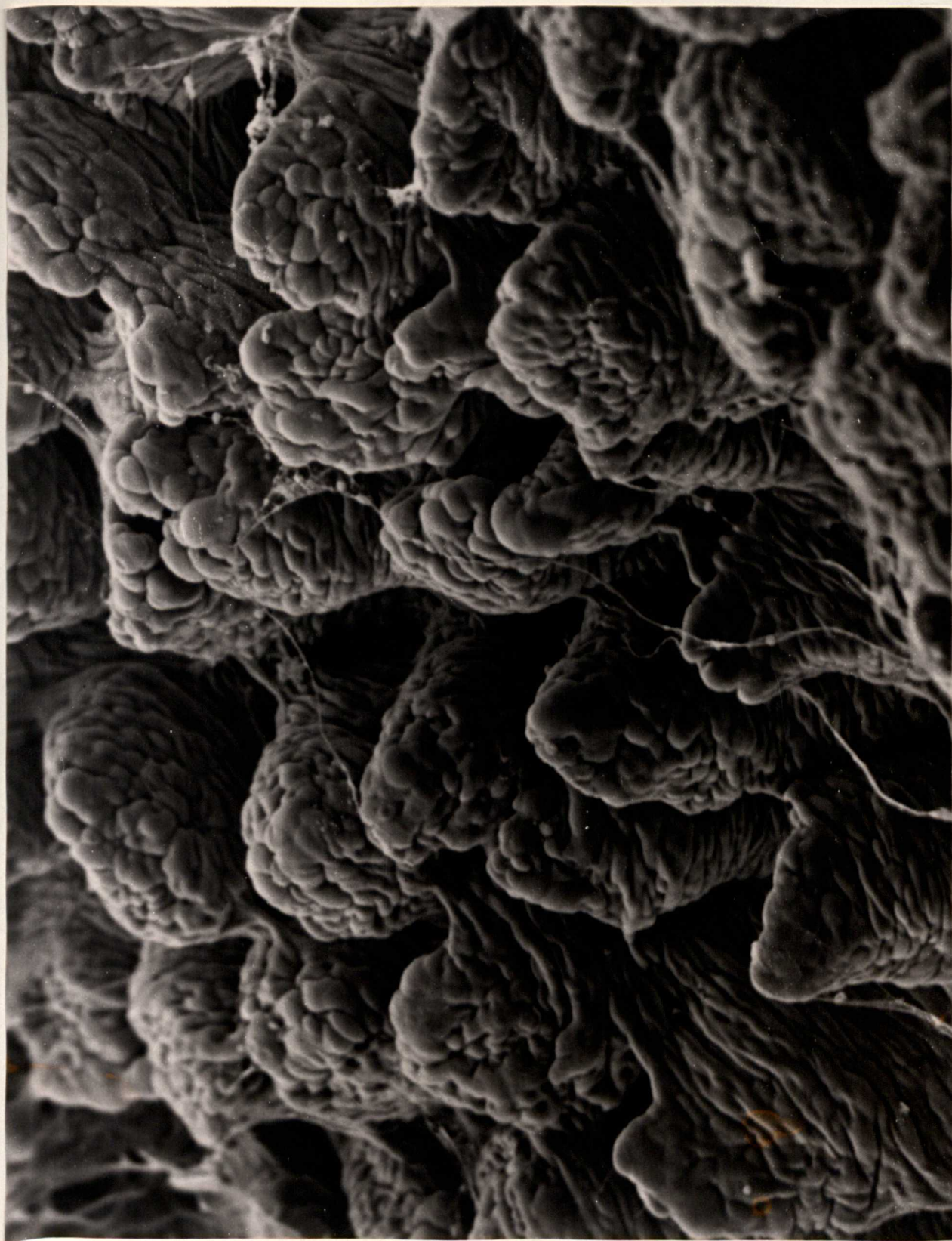


Fig. 83: Canine Parvovirus Infection - Jejunum of Dog 33.

The villi are shortened and irregular in height. Epithelial fusion between adjacent villi is apparent (arrow) and in one area several fused villi have formed a large irregular protuberance on the mucosal surface. Taken at 7 DPI.

x 480.

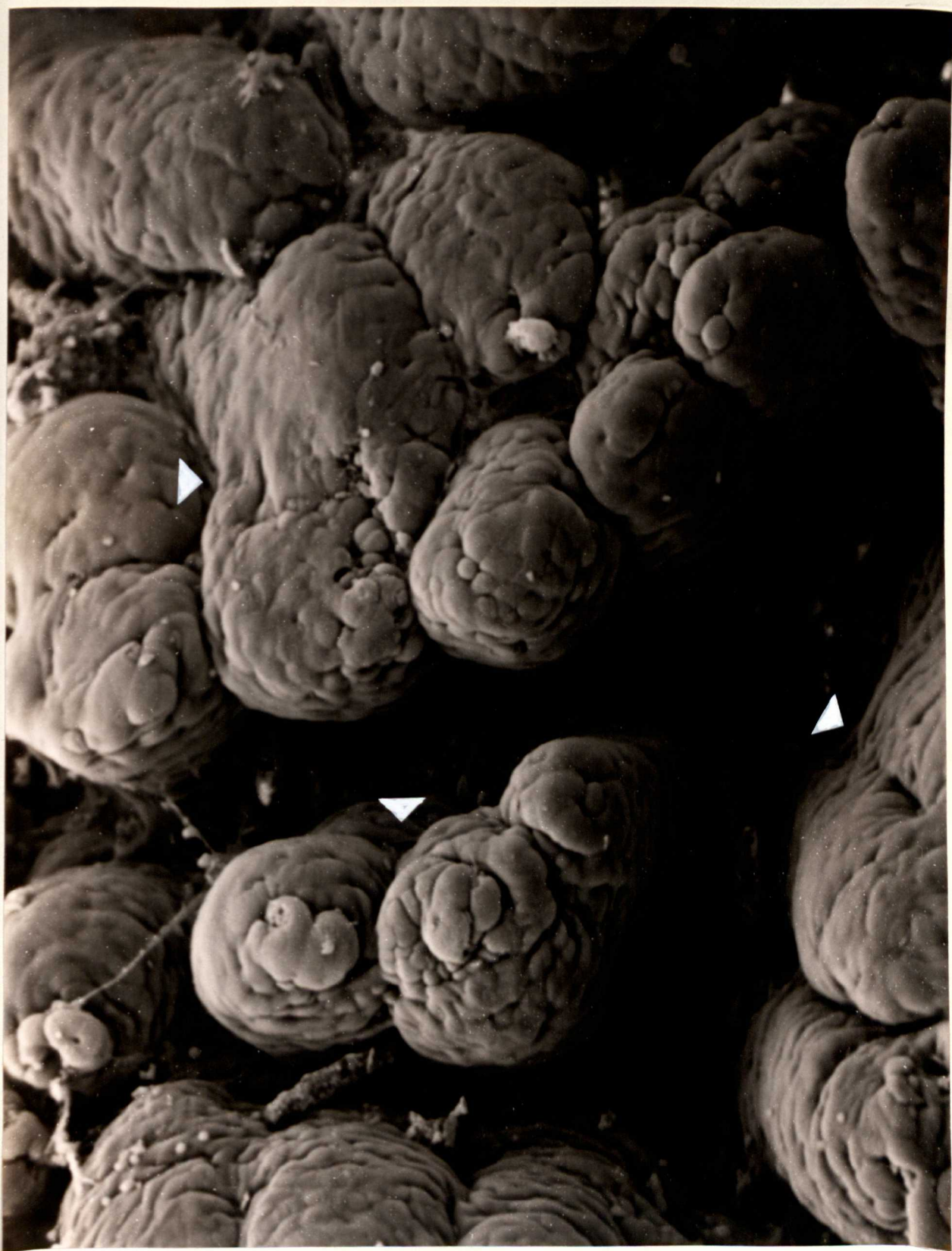


Fig. 84: Canine Parvovirus Infection - Jejunum of Dog 34.

Slender finger-like villi are apparent although they are irregular in height. The apices are irregular in shape and on some villi are pointed (arrow). Taken at 8 DPI.

x 480.



CHAPTER 8 : FINAL DISCUSSION
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The studies described in the preceding chapters of this thesis have resulted in the reproduction of enteric disease in pups following oral infection with CPV and have elucidated the pathogenesis of infection. The method of production of the oral challenge which was developed appeared satisfactory and the immunocytochemical techniques developed for the demonstration of CPV antigen should have future applications both to diagnostic work and to investigation of other disease syndromes, especially myocardial disease, which may be associated with CPV infection.

One of the most striking findings in this study, as in others (Pollock, 1982; Carmichael et al., 1980), has been that clinical enteric disease has not been the invariable sequel to infection with CPV. The initial systemic phase of CPV infection, with viral replication in lymphoid tissues and the production of viraemia appeared to be constant features. Since excretion of virus from the small intestine also occurred almost invariably, localisation of virus to intestinal mucosa would again appear to be consistent. The variable factor is the degree of damage which occurs in the intestine following viral localisation and which determines whether or not clinical evidence of enteric disease will result. Thus, although the appearance of enteric disease is intimately related to the pathogenesis of the infection, there would appear to be other factors involved which, in large part, determine the eventual outcome.

Following oral infection with CPV, virus localises rapidly in the lymphoid tissues with antigen detectable in thymus 24 hours after infection. Precisely how virus reaches the lymphoid tissue from the alimentary tract is still unclear. Absorption through the pharyngeal lymphoid ring has been suggested (Potgeiter et al., 1981). This route has also been postulated for FPV in the cat (Gillespie and Scott, 1973) although this has never been confirmed. In the present study, viral antigen was demonstrated first in the thymus (at 1 DPI) and only subsequently (at 2 DPI) in the peripheral lymphoid

tissues including the palatine tonsil and retropharyngeal lymph node. This pattern of spread would argue against the 'pharyngeal' theory.

However the inherent resistance of CPV to low pH (Carmichael and Binn, 1981) would allow passage of ingested virus through the stomach into the small intestine where adsorption to intestinal epithelial cells could occur in the small intestine, the normal active secretion of fluid from the intestinal crypts (Nasset and Ju, 1973) would tend to prevent intestinal contents containing CPV from directly contacting and infecting the susceptible crypt enterocytes. Certainly, there was no evidence from the present studies that direct invasion of intestinal crypt cells could occur: viral antigen was detected in these cells only from 4 DPI. However, non-destructive absorption of virus through the villi into the lacteals or blood vessels could, in theory, occur, leading to localisation in lymphoid tissues. Viral antigens were not detected in either the epithelium or lamina propria of the villi at 1 DPI in the present study. However, since virus was already present in the thymus at 1DPI, adsorption must have occurred before this time. In any case, it is likely that only very small amount of virus would be present in/on any individual villus which would have been beyond the sensitivity of immunocytochemical techniques. Detailed studies during the initial 24 hours after infection would be needed to determine the precise route of infection.

Replication of virus in the lymphoid tissues with lymphocytolysis and depletion is the main finding in the first few days following infection. Viral antigen and lymphocytolysis were found in greatest amounts in the thymic cortex and germinal centres of lymph nodes, reflecting the propensity of CPV for mitotically active cell populations. The very extensive lymphocytolysis found in the thymic cortex, compared to the other lymphoid tissues, is probably also a reflection of the extremely high mitotic rate which is found in this organ (Everett and Tyler, 1970).

In the study described in Chapter 3, lymphocytolysis was not observed until 3DPI whereas, in Chapter 7, it was already established by 2DPI. A much greater challenge was administered in the latter study and the apparent earlier appearance of destructive lymphoid lesions could be a dose related effect. Alternatively, this apparent difference could be ascribed to natural biological variation.

The depletion of thymus and lymph nodes as a result of lymphocytolysis is identical to the findings in FPV infection in cats, (Langheinrich and Nielson, 1971). The large "histiocytic" cells with copious pale staining cytoplasm found in the depleted germinal centres of lymph nodes, spleen and GALT were first described as "hypertrophied monocytes" in cats with FPV (Hammon and Enders, 1939a). Lawrence et al. (1940) suggested these cells were of reticuloendothelial origin and there seems little doubt that these are the members of the mononuclear phagocyte system currently termed "histiocytic reticulum cells" (Miller-Hermerink and Lennert, 1978) or "dendritic cells" (Henry and Farrer-Brown, 1981). It has been stated (Miller-Kermelink and Lennert, 1978) that these cells do not proliferate and therefore the prominence which they assume in depleted B-cell areas may be due, not to active proliferation as suggested by Johnson et al. (1967) and Fowler and Rohovsky (1970), but to the massive loss of surrounding lymphocytes making the histiocytic cells more obvious. Phagocytosis of lymphocyte debris by these mononuclear cells with increase in cytoplasmic mass may also facilitate their visualisation.

In the lymph nodes, spleen and GALT, regenerative changes appear rapidly after active lymphocytolysis with, in the nodes, early paracortical expansion and repopulation of germinal centres by 7 DPI and marked regeneration by 10 DPI. Regeneration in the thymus was much slower with, even at 10-13 DPI, still only a few dense foci of lymphocytes present in the depleted cortices. In mammals, thymic repopulation after irradiation induced depletion is primarily

from bone marrow although surviving thymic lymphocytes do have some limited regenerative capacity (Hiesche and Revesz, 1974). The sparse viral antigen detected in the bone marrow of a few dogs in the present study could represent viral replication in the marrow stem cells which would normally migrate and repopulate the thymus. Destruction of these stem cells could account for the apparent failure, or slowness, in thymic regeneration. Alternatively, the extreme degree of thymic destruction might simply mean that regeneration requires a much longer period of time. It is not known whether thymic regeneration would have occurred in the dogs in this study had they been kept for a longer period.

Severe thymic atrophy was perhaps the most consistent pathological lesion in infected dogs in this study. The severity was confirmed by quantitative studies on thymic/body weight ratios. In studies of vaccine efficacy, this quantifiable and persistent lesion would prove a useful parameter for estimation of viral replication in vaccinated and control dogs following challenge. This is especially pertinent in view of the inconsistency of clinical and pathological findings which have already been found in unvaccinated control animals in most vaccine studies (Chapek et al., 1980; Pollock and Carmichael, 1982a and b).

The appearance of a non-cell associated plasma viraemia at 3 and 4 DPI in the present study followed the development of lymphocytolysis and could well have been a direct result of it with the lymphoid cycle acting as an amplification system increasing the degree of viraemia. A similar, non-cell associated viraemia peaking at 3-4 DPI, was found by Pollock (1982) in oronasally infected dogs. Pollock (1982) also found a non-cell associated viraemia following intravenous infection but recorded both smaller amounts of virus and an earlier peak of viraemia in intravenously as opposed to oronasally challenged animals. There was also an earlier onset of clinical signs, viral excretion and antibody production in

parenterally challenged animals. Pollock suggested that his artificial parenteral challenge by-passed a step in the more natural oronasal infection, possibly one of primary viral replication in lymphoid tissues (possibly GALT). This initial replication in lymphoid tissues appears to be confirmed by the results in the present study. Interestingly, McAdaragh et al. (1982) in a limited and uncontrolled study of only four dogs, infected by dual oropharyngeal and parenteral routes, also found a time scale of events similar to that in Pollock's (1982) parenterally challenged dogs. McAdaragh and his co-workers, however, interpreted their findings as indicating that generalised viraemia was the initial stage in pathogenesis without considering that their parenteral route of inoculation could have influenced their findings.

In the studies described in this thesis, as in other investigations (Pollock, 1982; McAdaragh et al., 1982), cessation of viraemia coincided with the development of circulating antibody. Antibody was detectable at 5 DPI rising sharply to a plateau by 7 DPI. It is difficult to reconcile this rapid and high antibody response, also noted by other workers (Carmichael et al., 1980; Robinson et al., 1980b; Potgeiter et al., 1981), with the striking lymphocytolytic activity of the virus. The clonal selection hypothesis for production of antibody (McConnell et al., 1981) is based on the response of lymphocytes bearing specific surface receptors. On contact with antigen, lymphocytes with appropriate receptors divide to produce clones of effector cells including antibody producing plasma cells. However, with CPV infection, it might be expected that the initial contact between virus and lymphocyte which results in division would render the lymphocyte susceptible to infection and destruction by the virus.

In the face of this paradox, there are at least three possible explanations of the rapid humoral response. First, it may be that presentation of antigen to lymphocytes occurs only

after inactivation of virions, possibly by macrophages: antigenic stimulation will occur but there will not be simultaneous infection. Second, since defective particles are produced, some lymphocytes might be primed by defective, non-infectious virions. Third, the presence of specific receptors on the lymphocytes, while allowing stimulation, might prevent penetration and replication. Which, if any, of these explanations might be correct cannot be determined but the humoral response to CPV is undoubtedly one of the most striking features of the infection.

In contrast, given the extensive lymphocytolysis found in the dogs in the present studies, it is not surprising that, where reliable haematological data were available (in Chapters 6 and 7), infected dogs developed some degree of lymphopaenia. A degree of lymphopaenia was noted as early as 3 DPI, was more marked by 4 DPI and also present at 5-7 DPI in the serial study described in Chapter 7. In the investigation recorded in Chapter 6, where dogs were kept for a longer period, a reactive lymphocytosis was recorded from 7 DPI onwards. A reduction in total circulating lymphocytes, most marked at 4-5 DPI after oral infection with CPV has been noted by most investigators (Appel et al., 1979a; Carman and Povey, 1980; Robinson et al 1980b; Carmichael et al., 1981; Potgeiter et al., 1981). Lymphopaenia, most severe around 5 DPI, also occurs following FPV infection in the cat (Rohovsky and Griesemer, 1967; Rohovsky and Fowler, 1971). The large activated lymphocytes found on examination of blood smears in the dogs with reactive lymphocytosis from 7 DPI have also been observed in field cases of CPV enteritis. The circulating lymphopaenia probably simply reflects the destruction of lymphocytes in thymus, nodes, spleen and GALT. In the work of Appel et al. (1979a), lymphopaenia following infection was observed in dogs 1 year old as well as in pups. Since the thymus is greatly involuted by 1 year, the lymphopaenia may well be due in large part to destruction in non-thymic lymphoid tissues.

Although lymphopaenia was a finding in this study, in many dogs it was insufficiently severe to be reflected in an overall leukopaenia and was only noticeable on differential counts. Leukopaenia, when it developed, was associated with the development of neutropaenia but, in these studies, was not an invariable occurrence. Neutropaenia and leukopaenia occurred only in dogs with severe clinical disease. This correlation is also apparent in other reports of experimental CPV (Carman and Povey, 1980; Robinson *et al.*, 1980b; Potgeiter *et al.*, 1981) although only Potgeiter and his co-workers apparently noticed the relationship. This group of investigators noted that relative lymphopaenia was the only consistent haematological finding in infected dogs while neutropaenia occurred only in animals with clinical signs of enteric disease.

In contrast, overall leukopaenia with both lymphopaenia and neutropaenia is regarded as the classical feature of FPV infection in the cat (Kahn, 1978). Certainly, in severely affected cats there can be dramatic leukopaenia with total leukocyte counts as low as $200/\text{cm}^3$ ($0.2 \times 10^9/\text{litre}$) being recorded (Riser, 1943, 1946). Riser attributed the neutropaenia to bone marrow aplasia associated with viral damage. Kahn (1978), quoting the work of Rohovsky and Fowler (1971) also stated that the panleukopaenia with absolute neutropaenia and lymphopaenia of FPV infection could be attributed to viral replication in leukocyte precursors. However, Rohovsky and Fowler (1971) did not proffer any direct evidence for viral replication in myeloid cells although they did note a primary lymphopaenia with secondary neutropaenia in their germ-free experimental animals. The reality of this neutropaenia was difficult to evaluate in view of the paucity of neutrophils in germ-free animals.

In an earlier paper, Rohovsky and Griesemer (1967), also using germ-free animals, had noted that the leukopaenia which developed was due primarily to a severe absolute

lymphopaenia with neutropaenia being a secondary feature developing later in infection during clinical illness: in the bone marrow there was, paradoxically, an increase rather than decrease in myeloid elements. Similarly, in a study using conventional cats, Larsen et al. (1976) found the initial haematological change to be a lymphopaenia. These workers also demonstrated a neutropaenia but on studies of marrow found no morphological evidence of destruction of myeloid precursors, the main finding being depletion of mature neutrophils. There is, therefore, little if any evidence that in FPV infection in the cat viral replication in myeloid cells is primarily responsible for the development of neutropaenia.

In the studies described in Chapters 3, 6 and 7 above, evidence of only limited viral replication in bone marrow was found. At 5-6 DPI, sparse inclusion bodies and occasional solitary positively stained cells (by immunocytochemical techniques) were found. The main change noted in the marrow was marked depletion of mature neutrophils. Other workers (Robinson et al., 1980b; Potgeiter et al., 1981) have also noted depletion of the reserve marrow pool of mature neutrophils in severely-affected dogs following experimental CPV infection. The limited cell destruction in the marrow indicates that the depletion of mature neutrophils is due not to destruction of specific precursor cells but to withdrawal of the reserve pool to some other site.

Even in the normal dog (Perry, 1970) there is considerable loss of neutrophils into the intestinal lumen and this may be greatly increased by enteric disease. In the present study, there were striking enteric lesions and numerous neutrophils could be seen in the mucus overlying affected areas in all dogs with circulating neutropaenia and marrow depletion. This correlation would suggest that in CPV infection (and probably also in FPV infection) the development of neutropaenia is a secondary effect resulting from the development of enteric lesions with increased loss of neutrophils through the damaged

intestinal wall. In dogs with mild enteric lesions, withdrawal of cells from the marrow reserve could compensate for increased loss so that circulating neutropaenia would not necessarily be evident. However, in dogs with severe intestinal lesions, the marrow reserve would quickly have become exhausted and a circulating neutropaenia would result. The presence of an overall leukopaenia with neutropaenia would then indicate the presence of severe enteric pathology, justifying a poorer prognosis for such animals as suggested by Woods et al. (1980).

In the studies described in this thesis, excretion of virus in the faeces of infected dogs, as detected by assay of viral specific haemagglutinin, was detected in all but one of the infected dogs kept for 4 or more DPI when virus would have been expected to have reached the intestine. The exception was one dog (dog 7) infected with tissue culture virus in the preliminary study. The appearance of specific haemagglutinin in faeces at 4-7 DPI is in agreement with most other workers (Carmichael et al., 1980; Potgeiter et al., 1981; Pollock, 1982). Carman and Povey (1982a) detected virus in the faeces of 20 of 24 dogs at 6 DPI using virus isolation although only 4 of the 24 were positive by HA assay. This may indicate a relative insensitivity of HA assay as opposed to isolation. Since the dogs in Carman and Povey's study were severely ill, with dysentery, there could have been antibody in the intestinal content, as described in Chapters 6 and 7 above, which may well have interfered with the HA assay.

Pollock (1982) detected low levels of virus in faeces of infected dogs one day after oronasal challenge. The amount detected represented only 1/1000 of the original infecting dose and probably reflected passive transport of ^{The} challenge ^{organism} through the gut. Similar findings were reported by Carman and Povey (1982a). Only in the initial study recorded in Chapter 3 of the present series was viral antigen detected in faecal material collected at 1 DPI. It might have been expected, in view of the large challenge administered in the later studies of Chapters 6

and 7, that viral antigen would also have been detected in these studies. However, samples were not collected at 1 DPI in the study in Chapter 6 (owing to the fire) and in the investigation in Chapter 7, antigen was not detected. More frequent sampling of faeces over the initial 36 hours following infection might have detected passive transport of the challenge virus through the gut.

Although viral excretion and the development of an antibody response were consistent findings in this series of studies, clinical enteric disease was variable. Some infected animals showed no clinical evidence of enteric dysfunction while others had obvious intestinal disease varying in severity from a slightly loose faecal consistency through mucoid diarrhoea to profuse dysentery with vomiting and collapse. This variability in clinical response is apparent from examination of published accounts of experimental CPV infection (Appel et al., 1979a; Carmichael et al., 1980; Robinson et al., 1980b; Potgeiter et al., 1981; Pollock, 1982).

The clinical signs observed and the time course of their development i.e. dullness and anorexia at 3-4 DPI with diarrhoea and vomiting from 5 DPI, are in agreement with the results of Carman and Povey (1982a) and Pollock (1982) following oronasal challenge.

Marked pyrexia was not observed in any of the dogs in this study although there was a slight increase in temperature at 4-5 DPI, compared with pre-infection values for individual animals and values obtained in control dogs. Only occasional values were above the "normal maximum" for the dog of 39.5°C, the maximum temperature recorded being 40.1°C. A similar mild elevation in temperature has been recorded by other workers at 3-5 DPI (Pollock, 1982) 4-6 DPI (Carman and Povey, 1982a) and 3-5 DPI (Appel et al., 1979a) although Robinson et al. (1980b) found a more marked pyrexia (mean approximately 40°C) in some infected dogs. Pyrexia does not appear to be a striking feature

of infection.

Pollock (1982) has suggested that the mild elevation in temperature which he noted, peaking at 5 DPI, coincided with the appearance of circulating antibody and could be due to localisation of circulating antigen/antibody complexes but it seems equally likely that the mild pyrexia could be the result of release of endogenous pyrogen in association with lymphoid cell destruction.

The development and nature of clinical disease mirrors the extent and severity of intestinal damage. The production of intestinal lesions follows viraemia and the localisation of virus to the proliferative epithelium of the intestinal crypts at 4 DPI. Infected cells become pyknotic leading to the increase in amphophilic cells noted on conventional histology. Loss and breakdown of pyknotic cells releases free virus into the crypts which then can either infect any neighbouring crypt epithelial cells which are undergoing division or be excreted in the faeces.

The characteristic intestinal lesion in CPV infection (and FPV infection) - destruction of crypt epithelial cells with loss of villi, dilation of remaining crypts and, in extreme cases, collapse of intestinal mucosa - is morphologically identical to that produced by sublethal irradiation (Warren, 1942). Kent and Moon (1973) made a direct comparison between irradiation and FPV infection in the cat.

The initial insult in both parvovirus infection and irradiation is destruction of replicating cells in the proliferative zone of the intestinal crypts. The changes which may then develop appear to be consequences of the primary requirement to maintain epithelial integrity in the face of net loss of epithelial cells. Loss of epithelium arises not only from failure of production of new cells from the proliferative

zone but also from continued extrusion of effete cells from the apices of villi. Depending on the degree of net cell loss, more or less extensive changes are seen in intestinal structure. From the studies described in this thesis, it is possible to put forward an outline of the sequential changes which contribute to maintenance of epithelial integrity with increasing or continued epithelial cell loss.

The earliest change seen (at 4-5 DPI), apparently in response to early crypt damage, was a reduction in the height of intestinal villi which would have reduced the surface area to be covered by the available epithelium. Where viral induced cell loss proceeded beyond the capacity of shortening of villi to accommodate reduced epithelial area, there was attenuation of the epithelium lining the crypts, (5-6 DPI) thereby increasing the surface area covered by individual epithelial cells. In severe case, where attenuation was present but apparently insufficient to maintain confluency, there was also reduction in depth of crypts and crypt dilation. The dilation may follow the physical principle that spheres can accommodate the greatest volume with the least surface area.

Large, cytomegalic epithelial cells were often seen in severe lesions with crypt dilation. Similar cells are observed following intestinal irradiation and have been interpreted as an aberrant attempt to maintain epithelial confluency (Kent and Moon, 1973) or as part of an early regenerative response (Warren, 1942). Certainly, regeneration of crypt epithelium does occur, being most striking in severe cases with increased cellularity and depth in the proliferative zone, overall elongation of the intestinal crypts and eventual reformation of villi. In irradiation studies, it has been shown (Leshner and Bauman, 1969) that the hyperplasia of the proliferative zone is due to repeated division of daughter cells from the proliferative zone which, under normal circumstances, would differentiate without further division. This increase in the proliferative pool is accompanied by an acceleration of the

generation cycle (Lesher and Bauman, 1969) which will further shorten the period required for reinstitution of normal structure.

Whether all the mechanisms which may maintain epithelial confluency will, in an individual animal, be brought into play will obviously be dependent on the severity of insult to the proliferative cells in the crypts. Where only a few cells are destroyed, only slight changes such as stunting of the villi may be observed whereas, if numerous cells are destroyed or destruction continues for some time, all the changes will develop. In the most severely affected animals, those found dead with dysentery, the various mechanisms described appeared to have been inadequate. In these dogs there was total loss of intestinal architecture with mucosal collapse and denudation of the intestinal surface, intestinal epithelium being represented only by basal, residual, quiescent, enterocytes and a few dilated crypts lined by flattened,

occasionally multinucleate cells. While it is possible that the collapse of the mucosa seen in these animals which died was the result of post-mortem artifact, similar changes were also seen in dog 9, killed in extremis, which would indicate this change was real. The sparing of basal cells at the very bottom of the

intestinal crypts was a noticeable feature in this study and has also been noted in irradiation damage (Kent and Moon, 1973). Sparing of these cells is probably associated with their low mitotic activity.

Despite the often severe epithelial damage and consequent disruption of intestinal architecture in the dogs studied in this thesis, there was surprisingly little in the way of acute inflammatory changes in the intestinal wall. Although slight congestion was noted, the main finding in the wall was oedema of the submucosa and muscularis. Oedema was particularly noticeable in dogs which had died with dilation, and microscopical examination, of the subserosal lymphatics which was presumably responsible for the thickening, rigidity and loss of elasticity of the intestinal coils. Similar intestinal oedema has been noted in sublethally irradiated rats (Warren,

1942) indicating that it is secondary to the mucosal lesions and is not a virus specific effect.

Sparse inflammatory cells, mainly macrophages with occasional neutrophils, were found in the lamina propria and submucosa of infected dogs but inflammatory cells were found in greatest numbers in the blanket of mucus which overlay affected areas of small intestine. This mucus, which caused problems in preparation of material for scanning electron microscopy, appears to be a consistent and integral component of the lesions of CPV infection. An apparent increase in the amount of mucus in faeces was noted in many of the dogs in this study and was also noted in the investigation of Carman and Povey (1982a). These workers postulated that the mucus observed in faeces had been discharged from goblet cells in response either to circulating antigen/antibody complexes or to the presence of antigen in the intestinal lumen of animals which were becoming sensitised to it. In the studies described here, there was no obvious correlation between the appearance of serum antibody (and the possibility of immune complexes) and the presence of mucus in faeces or on the surface of intestinal mucosa. The presence and thickness of mucus appeared to be directly related to the severity of the underlying mucosal lesion. In at least one dog, killed at 6 DPI, there were increased numbers of goblet cells in the epithelium of the ileum and a similar increase has been noted following sublethal irradiation (Friedman, 1945) but the precise origin and nature of the mucus remains unclear.

The cessation of cell destruction in the proliferative zone of the intestinal crypts is probably a direct consequence of the development of an immune response to infection with either leak of circulating antibody into the intestinal lumen or local production of antibody in the intestinal lamina propria. Certainly, antibody was detected in the intestinal content and faeces of several dogs in this study and coincided with

cessation of intestinal antigen detection by immunocytochemical techniques. Leak of serum antibody is likely to be particularly important in severely affected dogs with dysentery while local production may be more important in mildly affected dogs with minimal intestinal lesions. Exhaustion of the susceptible epithelial cell population by total destruction of cells in the proliferative crypt zones is an alternative hypothesis to explain cessation of viral excretion although this seems less plausible since, in many cases, areas of crypt regeneration with high mitotic rates may be seen adjacent to more acutely damaged crypts.

The severity of clinical signs following infection with CPV was directly related to the pathological changes. Dullness and anorexia, the most common clinical signs were found, to some degree in all infected dogs whether or not enteric signs supervened. Dullness and anorexia, occurring at 3-4 DPI were associated with the systemic phase of viral replication and lymphocytolysis.

The development and severity of enteric signs directly reflected the severity of intestinal pathology. Dogs with slight stunting of villi showed only mild or no clinical signs while advanced lesions with loss of villi and crypt dilation were associated with profuse diarrhoea. Complete collapse of the mucosa was found in the dysenteric animals which died. The cause of death in these animals was probably a combination of dehydration, electrolyte imbalance and circulatory collapse associated with loss of body fluids from the damaged intestinal mucosa.

In these studies, faecal excretion of virus was found in all but one of the infected dogs followed until 4-6 DPI. Localisation and at least limited replication of virus in intestinal epithelium must therefore occur in virtually all infected dogs. However, since not all excreting dogs subsequently develop clinical enteric disease, there must, as

mentioned at the beginning of this discussion, be factors other than simple viral localisation which govern the severity of intestinal lesions and, consequently enteric disease which develops after viral localisation to intestinal epithelium.

In view of the affinity of parvoviruses for actively dividing cells it is probable that the most important factor governing the development of severe intestinal pathology and clinical enteric signs following CPV infection is, as in FPV infection in the cat (Carlson and Scott, 1977a and b) the mitotic rate in the intestinal crypts at the time of viral localisation to this site.

Numerous factors are known to affect the mitotic rate in intestinal crypt epithelial cells including age, dietary change, intestinal flora or change in flora, irradiation and hormonal and neural factors (Leshner et al., 1964). Lactation (Cairns and Bentley, 1967), for example, is known to increase intestinal mitosis, presumably as a result of raised prolactin levels, while vagotomy in the dog (Silen et al., 1966) has been associated with increased epithelial cell turnover. Following irradiation, there is a marked increase in intestinal mitotic activity (Leshner and Bauman, 1969) as a regenerative response. The role of hormonal and neural factors in influencing the outcome of CPV infection is unknown and irradiation is unlikely to be involved except under experimental circumstances. Nonetheless, adult dogs infected accidentally with CPV while recovering from irradiation damage (Carmichael, personal communication) suffered morbidity and mortality far in excess of that normally seen in mature dogs.

The most important factors affecting intestinal crypt mitotic activity in the more general canine population are likely to be age, dietary changes and intestinal flora. While studies on the effects of these factors specifically in the dog are lacking, there have been numerous investigations into their effects in other species, particularly in rodents. It is likely

that the results of these investigations reflect general biological principles rather than species specific findings, since similar phenomena have been observed in those few studies carried out in other mammalian species.

It is difficult to strictly separate the possible effects of age, dietary change and intestinal flora, especially around the age of weaning when, in addition to factors associated simply with age, there will, of necessity, be changes in diet with concomitant alterations in intestinal flora.

However, it has been shown that, in general, there is a decrease in intestinal mitotic activity with increasing age (Thrasher and Greulich, 1964a and b), older animals having a more sluggish mitotic rate than young weaned individuals. The exception is the unweaned suckling in which the rate of intestinal cell proliferation is low and increases following weaning (Koldovsky et al., 1966). Weaning in the dog typically occurs at 5-7 weeks of age and this may be relevant to the high incidence of disease seen in pups at 7-8 weeks of age. Interestingly, Robinson et al. (1980b) simultaneously infected two litters of pups aged 4 weeks and 7 weeks with CPV : they found that disease was more severe in the older litter. Although the authors did not mention weaning or specify the diets of the animals involved, it is highly likely that the 4 week old animals were still receiving a predominantly milk diet.

Once weaning has taken place, it might be expected that CPV infection would be more severe in younger pups with higher intestinal mitotic activity than in older pups or adults. This is supported by the results in this thesis when the 8 week old puppies, used in Chapter 6, developed a much more severe clinical syndrome with high morbidity than was found in the 10 and 12 week pups studied in Chapters 7 and 3 respectively. Similarly, Appel et al. (1979a) did not find any clinical signs of disease following oral infection of adult dogs although the same group of workers (Carmichael et al., 1980) did

note unspecified clinical disease in pups orally infected with comparable doses of virus.

Alterations in dietary regimes are known to affect intestinal crypt mitotic activity. Starvation is associated with a slowing of cell replication (Sprinz, 1971) while refeeding following starvation results in a surge of intestinal mitotic activity (Altmann, 1972; Aldewachi et al., 1975). The development of a post starvation mitotic surge may have been responsible for the severity of clinical disease observed by Carman and Povey (1982a) when they infected dogs with CPV following a regime of starvation and refeeding, feeding being recommenced 3 days after challenge at the time when viral localisation to intestinal crypts might have been expected. Initial voluntary starvation due to dietary preferences may also play some role in the severe clinical disease which has been observed in boarding kennels (McCandlish et al., 1981).

The presence of intestinal flora has been shown to increase the mitotic activity of the small intestine (Sprinz, 1971; Leshner et al., 1964; Abrams et al., 1963) when compared to germ-free individuals and this is likely to have an important effect on the outcome of parvovirus infections. In FPV infection in the cat Carlson and co-workers (1977a and b) specifically demonstrated more virus infected cells and lesions in specific pathogen-free kittens than in germ-free kittens, the SPF cats having a crypt cell proliferative rate 2.24 times that of the germ-free animals. The dramatic changes in intestinal flora which occur at weaning (Hirsh, 1980) may be in large part responsible for the increase in intestinal mitotic activity which is noted at this time and which may render recently-weaned pups more susceptible to severe enteric disease following CPV infection. Change in diet in older animals, possibly also altering the balance of normal intestinal microflora, and thus intestinal mitotic rate, might also be a factor in the apparently increased severity of disease seen in boarding kennels.

Another factor which may well be important in determining the outcome of CPV infection is the presence of intercurrent intestinal disease. Infections which damage the epithelia of villi will cause increase in crypt mitotic activity and decrease in generation time (Sprinz, 1971) to replace epithelial loss. Consequently, other pre-existing or concurrent infections which damage luminal cells may increase the severity of disease following infection with CPV. Pollock (1982) reported increased severity of CPV infection in animals with *Giardia* infestations. Particularly in large kennel units, a variety of usually non-fatal viral, bacterial and parasitic problems may be present (Macartney, unpublished observations) and may contribute to the high morbidity and mortality encountered in such units (McCandlish et al., 1981).

Numerous other factors, apart from those affecting crypt mitosis, may play some role in modifying the severity of clinical disease in CPV infection: these may include dose, route of infection and origin of virus. Many of these factors have already been considered at length in Chapters 3 and 4.

In view of the relative mildness of disease observed following infection with tissue culture virus in Chapter 3, it was decided to eliminate any factors, such as tissue passage and low dose which may have contributed to the mild disease found. It was hoped that the improvement in the quality of challenge which was attempted by the purification of wild virus from faecal material would enhance the pathological lesions and clinical syndrome following infection.

However, the subsequent studies in Chapters 6 and 7 appear to indicate that the importance of the quality of challenge may be less important than the status of the dogs being challenged. Evidence for this comes from a comparison of the experiments in Chapters 6 and 7 where dogs from the same source were orally infected with virus of faecal origin. More severe disease was encountered in the younger, 8 week old, dogs

of Chapter 6 than in the older, 10 week old, dogs of Chapter 7 despite the greater infecting dose ($14.5 \log_{10}$ TCID₅₀/ml. as opposed to $10.5 \log_{10}$ TCID₅₀/ml.) which was administered to the older animals. The mild disease observed in Chapter 3 may equally have been the result of infection in an even older, 12 week, age group.

The number of different factors which may be involved in determining the severity of enteric disease following CPV infection has not always been appreciated by investigators. For example, Pollock (1982) found less severe disease after oral infection in "SPF" as opposed to "conventional" dogs and attributed the difference in severity to the difference, if any, there was between his "SPF" and "conventional" animals which were stated to be healthy at the start of the experiment. He did not consider the possible role which may have been played by the fact that the conventional dogs were purchased from an outside source and moved to the same kennels as the resident SPF animals with the changes in diet and management regimes which this would have involved. Neither did he consider that the conventional dogs were aged only 8 weeks while the ages of SPF animals ranged from 8 to 24 weeks.

The sudden appearance of CPV infection in 1978 was perhaps the most important event in canine medicine since the discovery of the viral aetiology of distemper. CPV infection is now established as one of the major canine diseases and, for the foreseeable future, is likely to continue to cause enteric problems in susceptible adults and, more particularly, in weaned pups as their maternal immunity wanes. Despite the development of vaccines against CPV both derived from antigenically related FPV isolates (Chapek et al 1980) and inactivated (Pollock and Carmichael 1982b) and attenuated (Carmichael et al. 1981) CPV strains, the inherent resistance of CPV to destruction and its longevity are likely to prevent its elimination from kennels as can be achieved with the less resistant viruses of distemper and

hepatitis. The burden of infection which may develop in dog kennels, and the difficulty in inducing active immunity in pups with maternal antibody, are likely to result in continued disease problems even in kennels using effective vaccines (McCandlish et al., 1981).

The same problems of viral resistance and maternal immunity have been responsible for the paucity of experimental studies on the myocardial form of CPV infection. Most dog kennels have experienced CPV infection and therefore most breeding bitches are immune themselves and provide protection for their pups' during in utero life and in the immediate postnatal period which is the time of crucial importance in the development of myocardial disease (Lenghaus et al., 1980; McCandlish et al., 1981). Even if one can acquire a non-immune bitch, it is extremely difficult, given the current ubiquitous distribution of CPV, to maintain this status for a sufficiently prolonged period to infect her pups at appropriate times.

The studies described in this thesis, together with those of other investigators, have elucidated the pathogenesis and pathology of enteric CPV infection in the dog and have shed light on the factors other than the mere presence of virus which may be responsible for the development of severe clinical disease. A knowledge of these factors should be useful in modifying management practices to reduce both the incidence and severity of disease which, with vaccination, should lead to effective control of this infection.

It is a reflection of the advances which have been made in veterinary science that, while distemper was first recognised in the middle ages and its aetiology established in the early years of this century, the disease was not effectively controlled until the late 1950s and early 1960s; in contrast, CPV infection, first arising in 1978, has already been defined and methods of control established.

CONCLUSIONS

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The eruption of severe disease caused by a new canine parvovirus which occurred in 1978 was the most significant event which has occurred in canine medicine in recent years. The studies described in this thesis have elucidated the pathogenesis of the enteric disease caused by CPV and have indicated which factors other than the simple presence of virus in susceptible dogs may contribute to the development of severe disease.

The pathogenesis of CPV infection reflects the inherent requirement of parvoviruses for actively dividing cells. Oral infection of weaned pups results in rapid viral localisation and replication in the mitotically active lymphoid tissues with lymphocytolysis and depletion. Virus is first detected in the thymus at 1 DPI and extensive, generalised lymphocytolysis is present by 3 to 4 DPI. In the thymus, destruction is so severe as to cause macroscopically evident thymic atrophy which persists for some time after infection.

Extensive multiplication in lymphoid tissues is followed by a non-cell associated plasma viraemia at 3 to 4 DPI which leads to localisation of virus in the mitotically active epithelial cells in the proliferative zone of the intestinal crypts. Excretion of virus in faeces, from 4 DPI onwards, follows localisation of virus to intestinal epithelial cells. A relative lymphopaenia is found at 4 to 5 DPI, coinciding with depletion of the solid lymphoid organs but this is not always sufficiently severe to be detectable on total leukocyte counts. Despite the extensive lymphoid lesions in infected animals there is a rapid and high circulating antibody response in infected dogs.

Following infection with CPV, the development of lymphoid lesions with relative lymphopaenia, the initial viral localisation to intestinal epithelia with excretion of virus and the development of circulating antibody are constant findings. In contrast, severe enteric pathology and clinical evidence of enteric disease develop only in a proportion of infected dogs.

The initial event in the development of enteric lesions is destruction of epithelial cells in the proliferative zone of the intestinal crypts. A series of changes then occur (from 5 DPI onwards) in intestinal structure apparently as an adaptive response to maintain epithelial integrity in the face of decreased epithelial cell production. These changes include shortening or even loss of villi, flattening of epithelial cells, especially in intestinal crypts, and dilation of intestinal crypts. Loss of surface absorptive area is likely to result in diarrhoea. When these mechanisms are insufficient to maintain epithelial integrity there is total mucosal collapse with superficial necrosis, dysentery and death. In less severely affected animals, there is hyperplasia of epithelial cells in intestinal crypts and reinstitution of normal structure and function. Within the small intestine, lesions appear first and progress most quickly in the anterior small intestine. There is no active gastritis or viral localisation and destruction in the gastric epithelium. Limited viral replication does occur in the colon but only minimal histological lesions develop.

Cessation of excretion of virus from the intestine is associated with the detection of specific antibody in intestinal contents; this could be the result of leakage of serum antibody from the systemic circulation, particularly in severely affected dogs, or of local production of antibody within the intestinal mucosa.

In severely affected animals there is, in addition to an initial lymphopaenia, a neutropaenia during the phase of clinical illness. Neutropaenia is due mainly to withdrawal of both circulating neutrophils and the marrow reserve of neutrophils to the damaged intestinal tract. There is only a limited viral replication in the bone marrow.

The main factor governing the development of severe enteric pathology and severe clinical disease following infection with CPV is the degree of mitotic activity in epithelial cells in the intestinal crypts at the time of localisation of virus to this site. If the mitotic rate is high, many epithelial cells will be destroyed and severe disease will result. If the mitotic rate is low, there will be only limited cell destruction within the adaptive capacity of the intestine.

Of the many factors which affect intestinal mitotic activity, age and changes in dietary regime and intestinal flora are possibly the most important. A combination of these factors is probably responsible for the high morbidity and mortality encountered when CPV infection occurs in recently weaned pups. Management or modification of such subsidiary factors could contribute to reducing morbidity and mortality due to CPV infection.

In view of the difficulty of consistently reproducing severe enteric disease following CPV infection, particularly in well-managed, older pups, there could be some difficulty in evaluating the efficacy of vaccines against CPV infection. The consistent, destructive lymphoid lesions with lymphopaenia which occur in the initial stages of infection in susceptible dogs and particularly the severe and quantifiable destruction of thymic tissue which is found would appear to be the most reliable indicators of viral infection to monitor in vaccinated and control dogs in experimental studies of vaccine efficacy.

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